Thyroid physiology in fish

Integration of neuroendocrine pathways in the control of thyroid gland activity in common carp

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Chapter 1

General introduction

The thyroid gland, composed of thyroid follicles, is the single site of biosynthesis of thyroid hormones in vertebrates. The synthesis of thyroid hormones involves the coupling of iodide to the phenolic ring of tyrosine residues in thyroglobulin. This organification of iodide in thyroglobulin is located extra-cellularly in a protein-filled matrix, the colloid, which is completely enclosed by thyrocytes. The two major thyroid hormones secreted by the thyroid gland are thyroxine (T4, 3,5,3'5'-tetraiodothyronine) and, to a much lesser extent, T3 (3,5,3'-triiodothyronine). Clearly, thyroid hormone biosynthesis depends on iodine availability; in fact, thyroid hormones are one of the few biological compounds in which iodine is incorporated. For thyroid hormone biosynthesis to rely on a trace element at first seems strange, not in the least when we consider the many fundamental processes under control of thyroid hormones, which will be addressed in the following paragraph. However, as we will see, the thyroid gland is very effective in the clearance of iodine from the blood and an adequate amount of iodine can be extracted from water or diet by aquatic and terrestrial vertebrates, respectively.

The thyroid endocrine system is highly conserved throughout evolution and has been described in all vertebrate species investigated thus far. Not only the actions of thyroid hormones, but also the processes and mechanisms that constitute the thyroid endocrine system are very similar. The biosynthesis of thyroid hormones, the central regulation of thyroid gland activity, peripheral metabolic pathways of thyroid hormones and thyroid function, are very similar in fish, amphibians, reptiles, birds and mammals.

Thyroid hormone-related activity has also been described in organisms lacking an anatomically well-defined thyroid gland. Thyroid hormone synthesis has been demonstrated in the endostyle of urochordates and cephalochordates, while in other evertebrates (*e.g.* echinoderms, arthropods, molluscs, cnidarians, poriferans) thyroid hormones are present, or biological effects of exogenously administered thyroid hormones are known (Eales 1997). Even bacteria (*Escherichia coli*) and plants have been shown to metabolise exogenous thyroid hormones and synthesise thyroid hormones, respectively. These observations emphasise the significance of thyroid hormones as important signalling molecules within an evolutionary context (Heyland and Moroz 2005).

Despite the similarities, differences in thyroid physiology have also been described among vertebrates. Comparative thyroid research in vertebrate species other than mammals, including fish, may reveal novel, unexpected mechanisms and concepts in thyroid physiology, which can provide a broader perspective for and increased insights in mammalian thyroid physiology.

Thyroid hormone functioning in vertebrates

The pleiotropic and pervasive actions of thyroid hormones can be ascribed to the plethora of genes that are regulated, positively and negatively, by thyroid hormones. For thyroid hormones to regulate gene expression, activation of the thyroid hormone-receptors is required. These nuclear receptors are expressed in virtually all tissues. Since the thyroid gland primarily secretes T4 and the nuclear thyroid hormone-receptor has a preference for T3 over T4, the conversion of T4 to T3 via extra-thyroidal deiodination is a crucial step in thyroid hormone functioning. The actions of thyroid hormones are numerous, but when broadly categorised mainly involve the regulation of developmental, growth and metabolic processes. The following paragraphs will address several functions of thyroid hormones which have been observed throughout the Vertebrata subphylum (Gorbman, et al. 1983).

Development

Thyroid hormones are key endocrine signals in the regulation of developmental processes, including metamorphosis and neoteny. Metamorphosis occurs in amphibians, *e.g.* the tadpole-to-frog transformation, and in fish, *e.*g. smoltification in salmonids and flatfish that transform from a symmetric pelagic larva to an asymmetric benthic juvenile. Gudernatsch was the first to demonstrate, in 1912, that exogenous thyroid hormones, administered in the form of horse thyroid, induced precocious metamorphosis in frogs (Gudernatsch 1912). Indeed, just prior to the metamorphosis climax, the thyroid endocrine system is activated in amphibians and fish (Power, et al. 2001; Tata 2006).

Growth

Thyroid hormones also play a primary role in the post-embryonic differentiation of tissues in vertebrates. For instance, thyroid hormones promote the differentiation of the endoskeleton, the integument and its derivates, the reproductive system, the nervous system and the digestive tract throughout the Vertebrata subphylum (Gorbman, et al. 1983). Normal growth of tissues is under the control of thyroid hormones, although their exact role is less clear. Thyroid hormones do not promote growth in all vertebrate species and the effects on growth are not direct but appear to be synergistic with that of other endocrine signals, *e.g.* growth hormone, insulin and insulin-like growth factor (IGF) (Laron 2003).

Metabolism

Thyroid hormones are probably best known for their effects on metabolic processes and are considered to be major determinants of basal metabolic rate. Over a century ago, Magnus-Levy was the first to associate thyroid hormones with the regulation of basal metabolic rate, as thyroid hormones stimulated oxygen consumption and body weight in man (Magnus-Levy 1895). Indeed, thyroid hormones have profound stimulatory effects on intermediary metabolism, *viz.* carbohydrate, lipid and protein metabolism. Additionally, thyroid hormones are a major determinant of non-shivering thermogenesis in endotherms, as they stimulate the uncoupling of the mitochondrial proton gradient from the production of ATP via uncoupling proteins (UCPs), resulting in the generation of heat (Kim 2008; Silva 2003). Interestingly, also in the ectothermic common carp (*Cyprinus carpio*), UCP expression is dependent upon metabolic status, *e.g.* temperature and nutritional level (Jastroch, et al. 2005), which may indicate an evolutionary ancient principle for the involvement of UCPs in the regulation of metabolic processes, other than thermogenesis, possibly the protection against reactive oxygen species that are formed during mitochondrial respiration. Also in ectothermic vertebrates, thyroid hormones have been implicated in the regulation of metabolic processes. In teleost fish, thyroid hormones increase oxygen consumption, exert gluconeogenic effects,

increase lipolytic enzyme activity and have anabolic effects on protein metabolism although negative and contradicting results have also been reported (see review by Plisetskaya, et al. 1983).

Thyroid physiology in fish

One of the first descriptive and comparative studies on the thyroid gland was conducted by John Simon in 1844. He observed and described the thyroid gland in numerous birds, reptiles and fish (Simon 1844) including the common carp that is the object of study in this thesis. Although at that time the function of the thyroid gland was largely unknown, Simon acknowledged the potential of comparative studies to further understand the physiology of the thyroid gland;

> *Since any attempt to illustrate the obscure physiology of these bodies [ductless glands] must of necessity be founded on a precise knowledge of their distribution in the animal kingdom, the deficiency* cannot be considered unimportant... (Simon, 1844)*
>
> * Deficiency in distribution (*viz*. absence) of thyroid tissue has not been observed in a vertebrate species thus far.

Studies on the thyroid gland in teleost fish continued in the 19th century (Baber 1881; Maurer 1886) and in the early 20th century (Gudernatsch 1911). Early experimental studies in fish thyroid physiology proved their value for clinical research. In 1914, David Marine investigated the occurrence and aetiology of thyroid hyperplasia in brook trout (*Salvelinus fontinalis*) in a hatchery (Marine 1914). He showed that a dietary factor, later identified as iodine, and not an infectious or contagious factor, was the main cause of the goitrous nature of the trout thyroid gland. This study, amongst others, formed the basis for his landmark pilot study on the effects of iodine administration to school children in Ohio (Marine and Kimball 1920), which eventually led to the global use of iodized salt to prevent cretinism and goitre, a practice that was also adopted in The Netherlands, as illustrated by the use of Jozo table salt.

Research on thyroid physiology in fish in the 20th century, was mainly stimulated by advances made in the field of clinical and mammalian thyroid physiology and not in the least by the progresses in biomedical techniques, *e.g.* immunological, biochemical, microscopy and molecular biological. Also, the growing economical importance of aquaculture worldwide resulted in an increased interest in fish endocrinology, including thyroidology. In the last decade of the 20th century thyroid research in fish increased considerably, a rise that could mainly be attributed to salmonid research by several Canadian research groups, in particular those of J. Geoffrey Eales in Manitoba and John F. Leatherland in Guelph.

The following paragraphs will address three aspects of teleostean thyroid physiology: 1) the activity and anatomy of the thyroid gland, 2) the central regulation of thyroid gland activity and 3) the biochemical characteristics of deiodinases.

The thyroid gland; activity and anatomy

In most vertebrate classes the thyroid gland is an encapsulated and mostly bilobed gland, located in the anterior neck, ventral to the larynx and pharynx. Like other endocrine glands, the thyroid gland is highly vascularised, which is mainly to support the secretion of thyroid hormones produced by the thyroid, as well as to support the supply of iodide and energy. Thyroid hormones are synthesised in thyroid follicles, the functional unit of the thyroid gland, which are composed of a monolayer of thyrocytes, enclosing an extracellular lumen filled with a colloid matrix (Fig 1a).

Thyroid hormone biosynthesis

Despite limited information on the biosynthesis of thyroid hormones in fish, there is little evidence to suggest that it would differ fundamentally from that in other vertebrates (Fig 1b).

A Na^+/I^- symporter (NIS) (Dai, et al. 1996) located in the basolateral membrane allows the thyrocyte to load iodide from plasma. The presence of a piscine NIS-homologue can be inferred from reduced accumulation of iodide

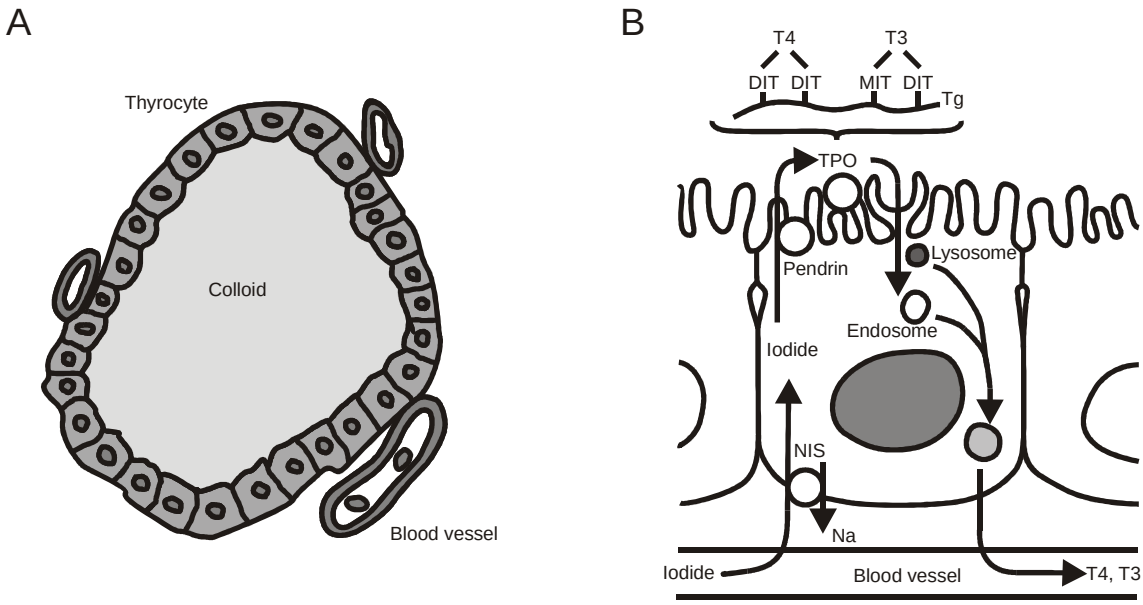

Figure 1. A. Cross-section of a thyroid follicle, showing the monolayer of thyrocytes that encloses the colloid. Blood vessels surround the thyroid follicle. **B.** Thyroid hormone biosynthesis. Iodide is transported through the thyrocytes into colloid where it is organified into thyroglobulin (Tg) and thyroid hormones are formed. After digestion of Tg in secondary lysosome, thyroid hormones are released into the blood.

after treatment with the goitrogen perchlorate (ClO_4^-) (Brown 1997), which is known to specifically block the NIS. A Cl^-/anion exchanger, pendrin (Scott, et al. 1999), located in the apical membrane is believed to extrude iodide into the colloid compartment (Yoshida, et al. 2002) where it is 'organified' into thyroglobulin. Thyroglobulin is a large (*ca.* 660 kDa) homodimeric glycosylated protein, which has been identified in teleost fish species (Baumeister and Herzog 1988; Kim, et al. 1984; Manchado, et al. 2008).

The extracellular organification of iodide occurs in three different processes, which are directly or indirectly mediated by thyroid peroxidase (TPO), located at the apical membrane of the thyrocyte. (*i*) TPO catalyses the oxidation of iodide (I^-) to iodonium (I^+). (*ii*) The reactive iodonium substitutes hydrogen atoms of tyrosine residues in thyroglobulin, to form mono- (MIT) and diiodotyrosines (DIT). (*iii*) TPO catalyses the coupling of two DIT molecules to form T4, and, to a lesser extent, of MIT and DIT to form T3. Treatment of fish with the TPO-inhibitors 6-*n*-propyl-2-thiouracil (PTU) or methimazole (MMI) induces hypothyroidism (De, et al. 1989; Elsalini and Rohr 2003; Van der

Geyten, et al. 2001; Varghese, et al. 2001) from which the presence of TPO in fishes may be inferred. Colloid resorption and micropinocytosis produce endosomes that fuse with primary lysosomes to form fagosomes. Endo- and exopeptidase activities hydrolytically digest thyroglobulin with the concomitant release of thyroid hormones, which are secreted across the basolateral membrane of the thyrocyte through an as yet unknown mechanism.

Heterotopic thyroid follicles

In virtually all teleosts and adult cyclostomes (lamprey and hagfish) the thyroid gland consists of rather diffusely scattered follicles (single or in small groups) in the region ventral to the pharynx, along the ventral aorta, where the branchial arteries branch off (Gudernatsch 1911). This is in contrast with the compact, encapsulated thyroid gland found in higher vertebrates. In several species of fish, thyroid follicles are not only present in the typical subpharyngeal region but are also found in other tissues such as the heart, spleen, liver, oesophagus, brain and choroid rete mirabile. The principal location for these heterotopic thyroid follicles is the head kidney (pronephros) and trunk kidney (opistonephros) (Baker-Cohen 1959). Reports on heterotopic thyroid follicles in renal tissue in a large number of species have appeared, with a surge of publications some three decades ago (Agrawala and Dixit 1979; Bhattacharya, et al. 1976a; Bhattacharya, et al. 1976b; Chavin 1956; Frisén and Frisén 1967; Joshi and Sathyanesan 1976; Lysak 1964; Olivereau 1960; Peter 1970; Qureshi 1975; Qureshi, et al. 1978; Qureshi and Sultan 1976; Sharma and Kumar 1982; Srivastava and Sathyanesan 1967, 1971a, b). Moreover, the absence of heterotopic thyroid follicles in several fish species has also been reported (Lysak 1964; Qureshi, et al. 1978; Qureshi and Sultan 1976).

Heterotopic thyroid follicles, when present, are active endocrine tissues as evidenced by their ability to accumulate iodine and synthesise MIT, DIT, and T4. These processes are sensitive to TSH as well as to thyrostatics (Bhattacharya, et al. 1976a; Chavin and Bouwman 1965). Furthermore, these activities in heterotopic thyroid follicles show a seasonal variation, similar to that of the subpharyngeal thyroid follicles (Bau and Parent 2000; Srivastava and Sathyanesan 1971b), indicating that heterotopic thyroid follicles are indeed

involved in the regulation of systemic thyroid hormones. It has been suggested that the development of heterotopic thyroid tissue may reflect a compensatory mechanism to iodine deficiency (Baker-Cohen 1959). However, it can be disputed whether natural hypothyroidism can occur in fish in an aqueous environment with a virtually infinite supply of iodine, even at trace concentrations of this element. Heterotopic thyroid follicles are likely a normal anatomical feature, and may reflect a phylogenetically more primitive anatomy, that works in concert with the sub-pharyngeal thyroid follicles to establish a healthy thyroid status in the animal.

One of the main actions of the head kidney is the production of the stress hormones cortisol by interrenal cells, and catecholamines by chromaffin cells. Moreover, the head kidney of fishes also harbours hematopoietic tissue that will provide different blood cell types and cells of the immune system. The juxtaposition of heterotopic thyroid follicles to these cells strongly hints at some paracrine relationship between these tissues and the thyroid hormone producing tissue. The head kidney may therefore facilitate interactions between thyroid-, stress- and immune signals.

Central regulation of the thyroid gland

In all vertebrates the activity of the thyroid gland is regulated by a negative feedback system, the hypothalamo-pituitary-thyroid axis (HPT-axis). In short; hypothalamic factors stimulate the release of thyrotropic factors from the pituitary gland, which again stimulate the release of thyroid hormones from the thyroid gland. Plasma thyroid hormones negatively feedback on the release of hypothalamic and pituitary thyrotropic factors.

As in mammals, thyroid-stimulating hormone (TSH, or thyrotropin), secreted by the pituitary pars distalis, is considered the key thyrotropic factor in teleost fish, since it elevates plasma T4 *in vivo* (Bandyopadhyay and Bhattacharya 1993; Brown, et al. 1985; Brown and Stetson 1983; Byamungu, et al. 1990; Grau and Stetson 1977; Inui, et al. 1989; Leatherland 1987; Specker and Richman 1984) and increases the release of T4 *in vitro* from thyroid follicle containing tissue (Grau, et al. 1986; Jackson and Sage 1973; Okimoto, et al. 1991; Swanson, et al. 1988). Furthermore, the expression of pituitary TSH β-subunit

mRNA is down-regulated by T4 and T3 in teleost fish, indicating a negative feedback (Chatterjee, et al. 2001; Larsen, et al. 1997; Pradet-Balade, et al. 1999; Pradet-Balade, et al. 1997; Sohn, et al. 1999; Yoshiura, et al. 1999).

Thyrotropin-releasing hormone (TRH), a hypothalamic amidated tripeptide (pGlu-His-Pro-NH_2) is the principal regulator of pituitary TSH cells in mammals. In several species of fish, TRH has been shown to up-regulate TSH-β subunit mRNA expression or to increase plasma T4 levels (Chatterjee, et al. 2001; Chowdhury, et al. 2004; Eales and Himick 1988; Han, et al. 2004). However, the thyrotropic effects of TRH differ greatly among species. In arctic charr (*Salvelinus alpinus*) low doses (≤0.1 μg/g body weight) of TRH elevate plasma T4 levels, but rainbow trout (*Oncorhynchus mykiss*) responded only to higher doses (1μg/g) (Eales and Himick 1988).

In several fish species TRH has been reported to be without effect on TSH release from pituitary cells or on the thyroid status of the animal, whereas the releases of growth hormone, prolactin or α-MSH were stimulated (Gorbman and Hyder 1973; Kagabu, et al. 1998; Lamers, et al. 1994; Larsen, et al. 1998; Melamed, et al. 1995; Peter and McKeown 1975). Moreover, TRH was shown to have an inhibiting effect on the thyroid system in several species of fish (Bhattacharya, et al. 1979; Bromage 1975; Bromage, et al. 1976; Peter and McKeown 1975). Although differences in experimental design, *e.g.* mode and dose of TRH administration and choice of experimental readouts, may account for some of the observed differences in TRH activity, it is apparent that the thyrotropic action of TRH in teleost fish remains equivocal and that TRH may be a misnomer for the piscine tripeptide pGlu-His-ProNH_2.

Corticotropin-releasing hormone

Another key hypothalamic factor involved in pituitary regulation is corticotropin-releasing hormone (CRH). In fish the CRH-immunoreactive hypophysiotropic neurons originate from the hypothalamic preoptic nucleus (nucleus preopticus, NPO) as do the hypophysiotropic TRH neurons.

The classical action of hypothalamic CRH in vertebrates, including teleosts, is the regulation of the release of adrenocorticotropic hormone (ACTH)

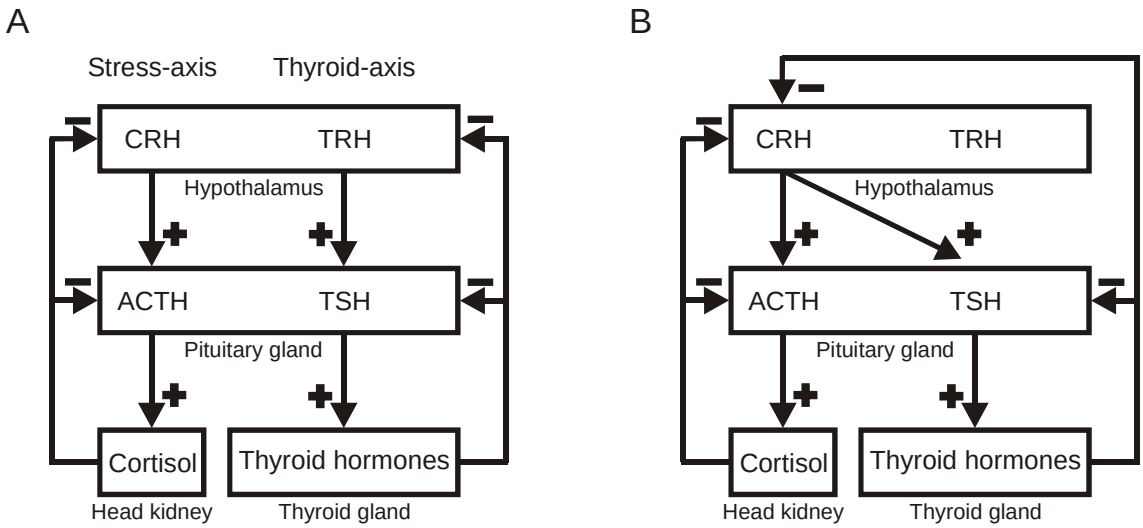

Figure 2. A. Simplified representation of the general vertebrate stress- and thyroid-axis. **B.** Hypothetical alternative for stress- and thyroid-axis in non-mammalian vertebrates, resulting in the integration of both signals.

from the pituitary pars distalis, which in turn stimulates the secretion of cortisol from the head kidney's interrenal cells during a stress response; the hypothalamo-pituitary-interrenal axis (HPI-axis) (Ando, et al. 1999; Flik, et al. 2006; Huising, et al. 2004; Metz, et al. 2004).

Hypothalamic CRH is also involved in other physiological responses, *e.g.* the regulation of food intake. Furthermore, CRH exerts a thyrotropic action in several non-mammalian vertebrate species, including teleost fish (De Groef, et al. 2006). Heterologous CRH and other members of the CRH family, *e.g.* urotensin I and sauvagine, are potent stimulators of TSH release from cultured pituitary cells from coho salmon (*Oncorhynchus kisutch*) (Larsen, et al. 1998). Intracerebroventricular (icv) administration of ovine CRH (oCRH) in fed goldfish (*Carassius auratus*) decreased total thyroid T4 and T3 content. In fasted goldfish icv oCRH treatment increased the free T4 and decreased free T3 contents of the thyroid (De Pedro, et al. 1995). These observations strongly suggest the involvement of CRH in the regulation of thyroid gland activity in these species.

It appears that, through an involvement of the shared signal molecule CRH, the corticotrope and thyrotrope axes in fishes are intertwined (Fig. 2) (see Kühn, et al. 1998 for other vertebrates). Possibly, thyroid hormones, of which

the release may be regulated by CRH, feedback on the NPO to modulate CRH bioactivity (Ceccatelli, et al. 1992; Dakine, et al. 2000) which would have a concomitant effect on the hypothalamus – pituitary – interrenal axis. This could also form a molecular basis for interactions between thyroid hormones and cortisol in fish. It has been observed that cortisol treatment reduces plasma T4 in European eel (*Anguilla anguilla*) and coho salmon (Redding, et al. 1986; Redding, et al. 1984), although stimulatory effects in rainbow trout and no effects in coho salmon have been observed as well (Leatherland 1987; Redding, et al. 1991).

Biochemical characteristics of deiodinases

Once secreted into the circulation, thyroid hormones are subject to a series of metabolising pathways which lead to major and minor iodothyronine metabolites. Two of these pathways concern sulfation (catalysed by sulfotransferases) and glucuronidation (catalysed by UDP-glucuronyltransferases) of thyroid hormones to yield conjugated thyroid hormones, and this has been reported for mammals (Visser 1996) and teleosts alike (Finnson and Eales 1996, 1997, 1998; Sinclair and Eales 1972). Conjugated iodothyronines are considered to be biologically inactive; their increased water solubility facilitates urinary and biliary excretion. The presence of glucuronidated and sulfated iodothyronines in fish bile and urine (Finnson and Eales 1996; Parry, et al. 1994) corroborates the role of hepatic conjugation as a clearance pathway.

Another major metabolising pathway is the deiodination of thyroid hormones. Deiodination involves the enzymatic removal of an iodine atom from the outer (phenolic) ring and/or inner (tyrosyl) ring of the iodothyronine molecule. Outer ring deiodination of T4 is required to yield the most potently bioactive hormone T3. Inner ring deiodination of T4 and T3 results in the relatively inactive thyroid hormones rT3 and T2, respectively (Fig. 3). Although rT3 and T2 do not bind to the thyroid hormone receptor, non-genomic biological effects of rT3 and T2 have been reported (Moreno, et al. 2008). Because of the activating and inactivating properties of deiodinases, these enzymes play a crucial role in the regulation of the thyroid status. The

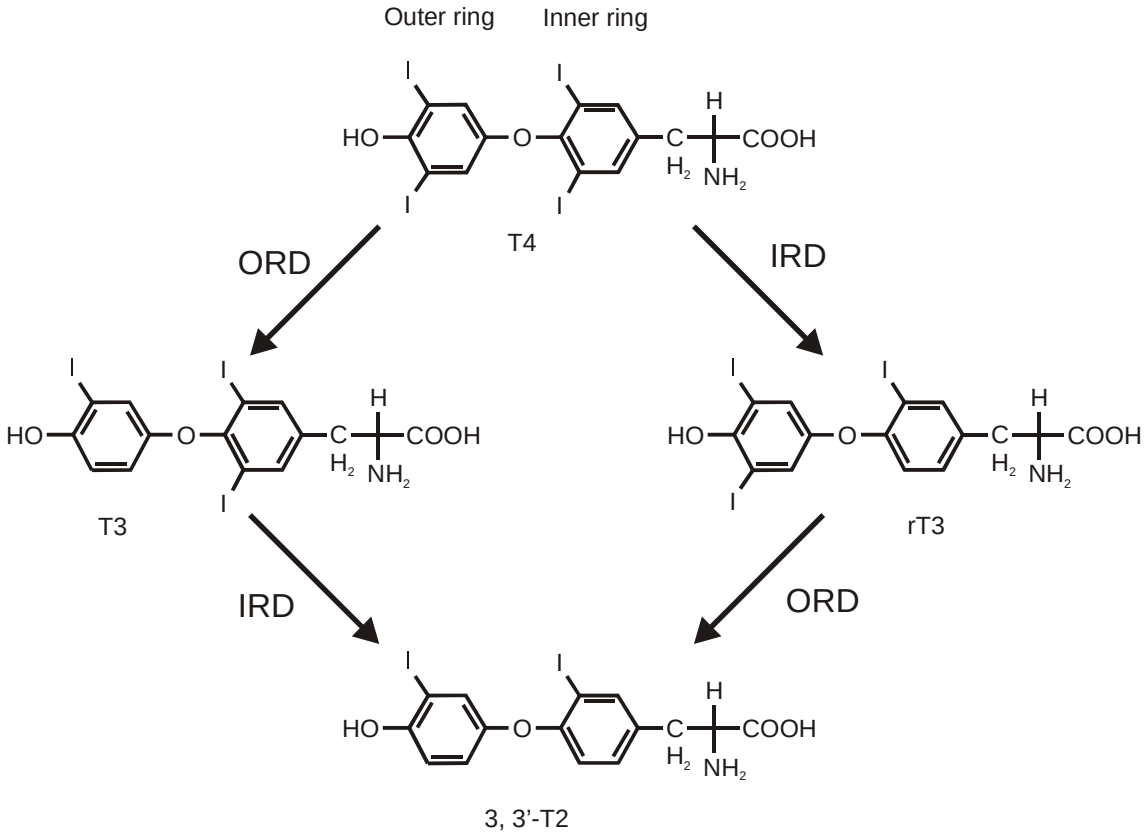

Figure 3. Pathways of subsequent outer ring (ORD) and inner ring monodeiodination (IRD) of T4.

differential expression and activity of deiodinases in peripheral tissues are therefore important determinants of plasma thyroid hormone levels. Deiodinases also regulate the intracellular thyroid status which is, to a high degree, independent of plasma thyroid hormone levels and tissue specific. Because of its pivotal role in regulating the peripheral thyroid status, deiodinase activity cannot be ignored when addressing the HPT-axis.

Three vertebrate iodothyronine deiodinases (D1, D2, D3) have been characterised, all containing a selenocysteine in the catalytic centre, a specific iodothyronine substrate affinity and tissue distribution, and preference for inner or outer ring deiodination (Bianco, et al. 2002; Gereben, et al. 2008; Köhrle 1999; Kuiper, et al. 2005) (Fig. 4). D1 and D2 possess outer ring deiodination activities, and are thus involved in the activation of the thyroid prohormone T4

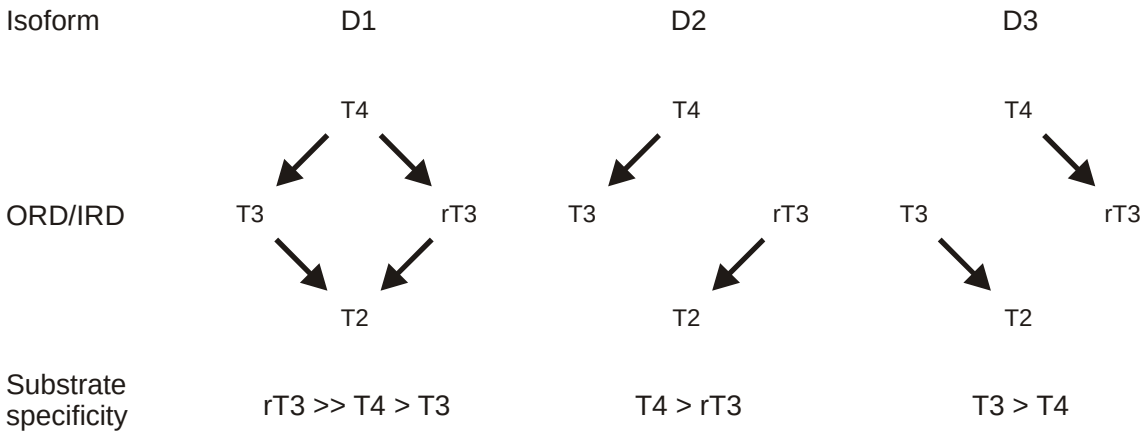

Figure 4. Mammalian characteristics (deiodination pathway and substrate specificity) of the three deiodinase isoforms.

to T3. D3, and D1 also, possess an inner ring deiodination activity whose role in thyroid hormone metabolism is the irreversible inactivation of T4 and T3.

Although deiodinases in some teleosts resemble their mammalian counterparts in their primary structure and biochemistry (Mol, et al. 1998), some peculiar biochemical differences exist among teleostean deiodinases (see Orozco and Valverde-R 2005 for a recent review). In a number of teleost species (*e.g.* gilthead seabream (*Sparus auratus*), Senegalese sole (*Solea senegalensis*)), D1 activity is inhibited by dithiothreitol (DTT), the thiol cofactor that, *in vitro*, potently activates mammalian deiodinases (Klaren, et al. 2005; Mol 1996). In the presence of DTT, teleost D1 is relatively insensitive to the thyrostatic PTU (Klaren, et al. 2005; Orozco, et al. 2003; Sanders, et al. 1997), as opposed to mammalian D1 which is inhibited by submicromolar concentrations of PTU. However, a PTU-sensitivity of teleost deiodinase is unmasked upon the removal of DTT from the incubation medium. Moreover, whereas the inner ring deiodination rates of sulfated T4 and T3 by rat D1 are greatly enhanced over those of native iodothyronines, no evidence was found for the deiodination of thyroid hormone sulfates in rainbow trout liver (Finnson, et al. 1999). Because of their unique biochemistry, investigations on teleost deiodinases may provide new insights on the working mechanisms of vertebrate deiodinases in general.

Objective and outline of this thesis

Because of their economic importance in Europe and northern America, salmonid fish species are well represented in endocrinological research in fish, including thyroid physiology. However globally, the aquaculture of salmonids only represents a mere 4.8% (in tonnes), whereas the lion share (44.5%) of the total worldwide aquaculture is determined by the rearing of cyprinid fish, of which common carp (*Cyprinus carpio* L.) alone represents 8.3% of the total global aquaculture (including aquatic plants, molluscs, crustacean, *etc*.) (FAO-UN 2007). Surprisingly, hardly any research has been performed on the thyroid physiology of this economically important fish species, particularly when considering the actions of thyroid hormones, *viz*. metabolism, growth and development, processes which are by no means trivial in aquaculture. Only three studies were performed on the functional aspects of the thyroid system of common carp (Kagabu, et al. 1998; Leray and Febvre 1968; Lysak 1964).

The objective of the research presented in this thesis is to investigate functional aspects of the thyroid endocrine system in common carp. This thesis will report on (i) the location and activity of the thyroid gland, (ii) the central regulation of the thyroid gland and the subsequent interaction with the stress response and (iii) the characterisation of extra-thyroidal deiodinases and their role in regulating thyroid hormone economy in fasting common carp.

To address the extent to which heterotopic thyroid follicles participate in the overall thyroid function in fish we describe in **chapter 2**, the location and the activity of the thyroid gland in common carp and, for comparison, Mozambique tilapia (*Oreochromis mossambicus*). Also the plasma dynamics and clearance of iodothyronines are described in both species. In **chapter 3** we attempt to describe the development of subpharyngeal and heterotopic thyroid tissue in early life stages of common carp through a histological analysis.

The central regulation of thyroid gland activity in common carp and the interaction with the stress-axis are described in **chapter 4,** in which we investigated the in vivo effects of experimental hyperthyroidism on hypothalamic, pituitary and peripheral mediators of the HPT- and HPI-axis. In **chapter 5** we describe direct central and peripheral interactions between the HPT- and HPI-axis in common carp by means of *in vitro* incubations with hypothalamic, pituitary and renal tissues.

In **chapter 6** we characterise 5'-deoidinase activity in liver and kidney of common carp. The biochemical and kinetic properties, as well as expression and protein levels are taken into account. In **chapter 7** the effects of different feeding regimens on the peripheral thyroid hormone system are described, with special emphasis on deiodination of thyroid hormones. The tissue-specific biochemical properties of deiodinase activity, as described in the previous chapter, are taken into account in the design of deiodination assays specific for liver and kidney.

In **chapter 8** the results obtained are summarised and discussed. Here, thyroid heterotopia, the interactions of the HPI- and HPT-axis, the integrative neuroendocrine control of thyroid gland activity and its functional implications on metabolic processes will be addressed.

The thyroid gland: a historic perspective

The thyroid related disorders goitre and cretinism have long been a familiar aspect of landlocked and mountainous societies around the world. Hippocrates (460-370 BC) and Galen (129-200 AD) already described a distinct tissue in the neck consisting of the salivary glands, the thymus and the thyroid body. It was not until the first detailed description of the separate thyroid gland by Andreas Vesalius in his monumental "De Humani Corporis Fabrica" (1543) that goitre was related to the thyroid gland. Thomas Wharton, in his "Adenographia" (1656), was the first to propose the name "thyroid gland", after the shape of the nearby thyroid cartilage, which resembles a *thyreos*, an ancient Greek army infantry shield.

Despite the increasing knowledge on the anatomy and structure of the thyroid gland that followed Vesalius' description, its function remained elusive for centuries. The thyroid gland was thought to lubricate the larynx, to heat the hyoid cartilage or to give mere shape and beauty to the female neck. The first physiological aspects of thyroid function were described by Robert James Graves in 1835 and Karl Adolph von Basedow in 1840; they associated goitre with tachycardia, exophthalmos and nervousness.

The concept of internal secretion by Claude Bernard (1855) in ductless glands, such as the thyroid gland, and the discovery that the thyroid gland contained iodide by Eugen Baumann in 1895, allowed for hypotheses that the thyroid gland synthesised and secreted an iodinated substance responsible for the reported physiological effects. Twenty years later, in 1915, Edward Kendall was able to isolate this substance, but it was Charles Harington who, in 1926, described the correct structure and named it thyroxine (T4, 3,5,3'5'-tetra-iodothyronine). It was not until 1952, that Rosalind Pitt-Rivers and Jack Gross discovered a second thyroid hormone, T3 (3,5,3'-triiodothyronine), which surprisingly appeared to be far more bioactive than thyroxine. The latter is now regarded a relatively inactive "prohormone".

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Chapter 2

Comparative thyroidology: thyroid gland location and iodothyronine dynamics in Mozambique tilapia (*Oreochromis mossambicus* Peters) and common carp (*Cyprinus carpio* L.)

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Abstract

In teleosts, the thyroid gland is mostly found in the subpharyngeal region. However, in several species thyroid follicles are found in, for example, heart, head kidney and kidney. Such heterotopic thyroid follicles are active, and considered to work in concert with the subpharyngeal thyroid. In Mozambique tilapia (*Oreochromis mossambicus*) thyroid activity is, indeed, restricted to the subpharyngeal region; in common carp (*Cyprinus carpio*) the functional endocrine thyroid is associated with renal tissues. The subpharyngeal follicles of carp comprise only 10% of the total thyroid tissue, and these follicles neither accumulate iodide nor synthesise or secrete thyroid hormones to a significant degree. Although the shape and size of carp subpharyngeal and renal follicles vary, the epithelial cell height of the thyrocytes and thyroxine immuno-reactivity do not differ, which suggests that the activity of the carp subpharyngeal thyroid follicles is dormant. Differences in thyroid physiology between the two fish species were further assessed at the level of peripheral thyroid hormone metabolism. Carp clears plasma of thyroid hormones faster than tilapia does. Furthermore, a significant amount of conjugated thyroid hormones was observed in the plasma of tilapia, which was preceded by the occurrence of thyroid hormone conjugates in the subpharyngeal region and coincides with the appearance of conjugates in the surrounding water. Apparently, plasma thyroid hormone conjugates in tilapia originate from the thyroid gland and function in the excretion of thyroid hormones. Our data illustrate the variability in teleostean thyroidology, an important notion for those studying thyroid physiology.

Introduction

The main products of the thyroid gland are thyroid hormones, the actions of which are pleiotropic and involve the regulation of metabolism, growth and development, including metamorphosis. Thyroid hormones are synthesised in thyroid follicles, the functional units of the thyroid gland, composed of thyrocytes enclosing a protein-filled colloid matrix. Thyroid-stimulating hormone (TSH) from the pituitary gland is the major stimulus for thyroid hormone synthesis and release (Blanton and Specker 2007; Eales and Brown 1993). Plasma thyroid hormone levels are not only determined by thyroid hormone synthesis and secretion, but also by peripheral metabolism (*viz*. deiodination and conjugation), clearance and excretion of thyroid hormones. Thyroid hormones are generally excreted as glucuronide or sulphate conjugates via the bile (Finnson and Eales 1996). Unlike the compact mammalian thyroid gland, the thyroid gland of most teleostean fish consists of non-encapsulated follicles scattered in the subpharyngeal region surrounding the ventral aorta (Gudernatsch 1911). In several species of fish, however, heterotopic thyroid follicles, *i.e.* follicles located outside the typical subpharyngeal region have been reported (Baker 1958).

Heterotopic thyroid follicles can be found near or in the heart, spleen, liver, oesophagus, brain and choroid rete mirabile of fish, but are generally restricted to tissues that ontogenetically derive from renal primordia, *viz*. the head kidney (pronephros) and the adult kidney (opistonephros) (Baker 1958). Thyroid heterotopia has been described in species throughout the Teleostei infraclass; it is found in representatives of the order of anchovies and herrings (Clupeiformes, 1 species), catfish (Siluriformes, 4 species), killifish (Cyprinodontiformes, 3 species), swamp eels (Synbranchiformes, 1 species), perch-like fishes (Perciformes, 3 species), rainbow fish and silversides (Atheriniformes, 1 species) and minnows and suckers (Cypriniformes, 14 species). Interestingly, 13 of the 27 fish species in which heterotopic thyroid follicles have been described belong to the family of carp and minnows (Cyprinidae), including species such as goldfish (*Carassius auratus*) and common carp (*Cyprinus carpio* L.).

Because of their ectopic nature, heterotopic thyroid follicles have often been interpreted as resulting from metastases (Berg, et al. 1953; Blasiola, et al.

1981; Nigrelli 1952). Although thyroid hyperplasia and neoplasia have been described in teleostean fish (Fournie, et al. 2005; Leatherland and Down 2001), normal heterotopic thyroid follicles do not follow the diagnostic criteria for thyroid hyperplasia, adenoma or carcinoma as proposed by Fournie, et al. (2005).

Most reports on heterotopic thyroid follicles in fish only describe the presence of heterotopic thyroid follicles without consideration as to quantitative or functional aspects (Agrawala and Dixit 1979; Baker 1958; Qureshi 1975; Qureshi, et al. 1978; Qureshi and Sultan 1976; Sathyanesan 1963). Extra-pharyngeal thyroid follicle populations have been reported to be less active than (Bhattacharya, et al. 1976), of equal activity to (Frisén and Frisén 1967) or more active than (Chavin and Bouwman 1965; Peter 1970; Srivastava and Sathyanesan 1971) the subpharyngeal thyroid tissue. The general opinion is that these heterotopic follicles work in concert with the subpharyngeal thyroid and contribute to the thyroid status of an animal.

Since iodide is exclusively incorporated into thyroid hormones and its metabolites, the use of radioactive isotopes of iodide offers unique possibilities for the investigation of thyroid hormone dynamics. Autoradiography of the thyroid gland in Mozambique tilapia (*Oreochromis mossambicus* Peters) and common carp serendipitously revealed differences in iodide metabolism, and pointed to the presence, in carp, of heterotopic thyroid tissue that is functionally different from that in the subpharyngeal region. This was the motivation for the studies described here.

Materials and methods

Animals

Common carp (*Cyprinus carpio* L.) of the all-male E4×R3R8 isogenic strain (Bongers, et al. 1998) were obtained from the Department of Fish Culture and Fisheries of Wageningen University, The Netherlands. Mozambique tilapia (*Oreochromis mossambicus* Peters) were obtained from laboratory stock. Fish were kept in 150 l tanks with aerated, recirculating city of Nijmegen tap water, at a photoperiod of 16 h light and 8 h darkness at 23°C for carp and 27°C for

tilapia. Fish were fed commercial fish food (Trouvit, Trouw, Putten, The Netherlands) at a daily ration of 1.5% of their estimated body weight. Animal handling followed approved university guidelines.

Whole body autoradiography

Juvenile carp and tilapia (standard length 6-8 cm) were exposed for 16.5 h to 250 μCi $Na^{125}I$, which was added to the aerated water in a 3 l tank, at 23 and 27°C, respectively. Thyrostatic potassium perchlorate ($KClO_4$) was added at a concentration of 1 mmol l^{-1}, and its effect on ^{125}I uptake was compared to that in an untreated group. After exposure, fish were deeply anesthetised with 0.1% (v/v) 2-phenoxyethanol and killed by immersion in melting isoflurane (-70°C). Animals were embedded in 5% carboxymethyl cellulose and stored at –20°C and whole-body cryosections were obtained according to a method described by Rijntjes, et al. (1979). In short, a carboxymethyl cellulose block containing a specimen was mounted on the stage of a LKB 2250-PMV cryomicrotome (LKB, Stockholm, Sweden). Sections were collected with the aid of cellulose tape that was applied to the upper surface of the carboxymethyl cellulose block, and were freeze dried in the microtome for 24 h. Sections 30 μm thick were taken every 90 μm. Freeze-dried whole-body sections of the whole fish were placed on Biomax MR-1 X-ray film (Eastman Kodak Company, Rochester, NY, USA); films were exposed for 3 days at -70°C after which they were developed according to the manufacturer's protocol.

Injection procedure and sampling

Carp (102±14 g; *N*=24) and tilapia (117±17 g; *N*=24) were injected intraperitoneally (i.p.) with 20.3 μCi carrier-free $Na^{125}I$ (Amersham Biosciences, Amersham, Bucks, UK) per 100 g body weight. The ^{125}I specific activity was 82×10^{15} Bq mol^{-1} and the radiotracer was dissolved in 0.9% NaCl. After injection, fish were immediately transferred to individual tanks with 3.5 l aerated city of Nijmegen tap water. During the experiment, water was sampled and radioactivity was measured. Fish were sampled at set times after injection by adding 0.1% (v/v) 2-phenoxyethanol to the individual tank to induce

anaesthesia. Blood was sampled by puncture of the caudal vessels with a heparinised syringe fitted with a 23 G needle and plasma was collected after centrifugation at 4°C (4000 *g*, 15 min). The fish were then killed by spinal transection and selected organs and tissues, as indicated in the figure legends, were collected. All tissues and the remaining carcass were weighed and the volume and weight of total bile and the collected plasma sample were determined. The radioactivities of bile and plasma were measured in an LKB 1272 Clinigamma γ-counter (Wallac, Turku, Finland) and immediately thereafter subjected to Sephadex LH-20 chromatography (see below). All tissues were homogenised according to Chopra, et al. (1982) with an all-glass Potter-Elvehjem homogenisation device in ice-cold 0.1 mol l^{-1} Tris-HCl buffer (pH 8.7) added at 6 ml g^{-1} tissue. Total radioactivity of the homogenates was determined as described for bile and plasma. The carcass was microwaved for 3 min at 800 W and homogenised in a blender in 200 ml 0.1 mol l^{-1} Tris-HCl buffer (pH 8.7). The resulting total volume was assessed and the radioactivity of sextuplicate 1 ml subsamples was determined.

Histochemistry

The subpharyngeal region, head kidney and kidney of four carp and tilapia (39.3±0.5 g) were fixed in Bouin's solution for 24 h. Tissues were dehydrated in a graded series of ethanol, embedded in paraplast and sectioned at 7 μm. Every 140 μm two serial sections were collected and mounted on glass slides. Sections were stained with a modified Crossmon's connective tissue stain (Crossmon 1937) as follows: 1.3 g l^{-1} Light Green SF yellowish (Chroma-Gesellschaft, Stuttgart, Germany), 1.7 g l^{-1} Orange G (Searle Diagnostic, High Wycombe, Bucks, England) and 2 g l^{-1} acid fuchsin (Fuchsin S from Chroma-Gesellschaft) were dissolved in distilled water at 80°C. The solution was cooled to room temperature, and 1 g of phosphotungstic acid hydrate was added to a 50 ml volume, followed by 2 ml glacial ethanoic acid and 100 ml absolute ethanol. The final solution was filtered and stored. Crossmon's trichrome stain was followed by a haematoxylin counterstain. Using this procedure, the colloid in thyroid follicles stains bright orange-red, which facilitates digital analysis of images.

Immunocytochemistry

Serial sections were incubated with 2% H_2O_2 and 10% normal goat serum in ice-cold phosphate buffer to inactivate endogenous peroxidase activity and to block non-specific antigenic sites. Sections were then incubated overnight with a polyclonal antiserum against thyroxine (MP Biomedicals, Irvine, CA, USA) at a dilution of 1:5000. Then, sections were incubated for 1 hour with a 1:200 dilution of biotinylated goat anti-rabbit secondary antibody (VectaStain, Vector Laboratories, Burlingame, CA, USA) and incubated for 30 min with VectaStain ABC reagent. Antibody binding was detected with 0.025% 3,3-diaminobenzidine (Sigma, St Louis, MO, USA) in the presence of 0.02% H_2O_2.

Morphological data analysis

Sections were analysed with a Leica DM-RBE light microscope (Leica, Wetzlar, Germany). Each thyroid follicle in the section was digitally photographed at 20-times magnification. The colloid in every follicle was manually selected using Adobe Photoshop 7.0 software and quantified using MetaMorph 6 software (Universal Imaging, Downingtown, PA, USA). The epithelial cell height of three thyrocytes per follicle in five follicles per tissue per fish was digitally determined. The area, perimeter, maximal diameter, length and width of every single colloid cross-section were measured. The shape of the colloid was described with three dimensionless shape descriptors: form factor, roundness and aspect ratio, and were calculated as follows (Ponton 2006):

$$\text{Form factor} = 4\,\pi\,\text{Area} / \text{Perimeter}^2,$$

where Area and Perimeter are the measured area (μm^2) and perimeter (μm) of a colloid, respectively. The form factor expresses the evenness of the colloid's outline; as its value approaches 1, so the outline resembles more the outline of a circle.

$$\text{Roundness} = 4\,\text{Area} / \pi\,(\text{Maximal diameter})^2,$$

where Area and Maximal diameter are the measured area (μm^2) and maximal diameter (μm), respectively, of a colloid. A colloid with a maximum roundness value of 1 perfectly resembles a circle.

Aspect ratio = Maximal length / Maximal width,

the larger the aspect ratio, the more elongated the colloid is; a ratio of 1 corresponds to a perfectly circular colloid.

An initial analysis of frequency distributions revealed that form descriptors were not Gaussian distributed, and we therefore chose the mode as a descriptive statistic. To avoid subjective selection of bin width and endpoint, we determined the frequency distribution by kernel density estimation (Parzen 1962) using an add-in utility (version 1.0e) for Microsoft® Excel from the Royal Society of Chemistry (Thompson 2006).

In vitro incubations

Subpharyngeal tissue between the second and fourth gill arches, head kidney and kidney was dissected from 14 carp (61±15 g). Tissues were weighed and diced into approximately 1 mm^3 fragments and immediately transferred to a microtitre plate in 3 ml Hepes-Tris-buffered medium (pH 7.4) saturated with carbogen (95% O_2-5% CO_2) and allowed to recover for 1 h. Then, tissues were transferred to a clean plate in 3 ml of the aforementioned buffer and exposed to 10 mIU ml^{-1} bovine TSH (bTSH; Sigma) or saline vehicle. Tissues were incubated for 24 h at 23°C, after which the incubation medium was sampled. Total T4 (thyroxine, or 3,5,3'5'-tetraiodothyronine) in the medium was determined using a commercially available enzyme-linked immunoassay (Research Diagnostics, Inc., Flanders, NJ, USA) according to manufacturer's instructions. Thyroxine-spiked samples gave representative readouts.

Thyroid hormone extraction

Several different methods based on extraction with Tris-HCl buffer, ethanol, methanol, butanol or chloroform were tested. We found a combination of Tris-HCl buffer and chloroform to be the most efficient in extracting radioactivity. Homogenates from the subpharyngeal region, head kidney and kidney tissues were obtained from intraperitoneally ^{125}I-injected animals, as described above. Then, 25 mg of pancreatin (Merck, Darmstadt, Germany), suspended in 0.1 mol l^{-1} Tris-HCl buffer (pH 8.7), was added to 1 ml of tissue homogenate as described by Tong and Chaikoff (1957) which was then incubated for 17 h at 35°C. Chloroform (1.5 ml) was added and the incubate was vigorously mixed for 2 min and centrifuged at 4°C (4000 *g*, 15 min). The water phase was collected by aspiration and stored at -20°C until further analysis; 1 ml of 0.1 mol l^{-1} Tris-HCl buffer (pH 8.7) was added to the remainder of the chloroform/pancreatin mixture, and it was mixed for 10 min, and incubated for 48 h at 4°C. The mixture was then centrifuged at 4°C (4000 *g*, 15 min) and the water phase was aspirated and stored at -20°C until further analysis. The extraction procedure was repeated once, after which the radioactivity of the three pooled water phases and of the remaining chloroform/ pancreatin mixture was determined.

Sephadex LH-20 column chromatography

Sephadex LH-20 column chromatography was performed as described by Mol and Visser (1985). In short, glass pipettes were filled with 1 ml Sephadex LH-20 (Amersham Biosciences, Uppsala, Sweden) suspension in water (10% w/v) and equilibrated with 3 × 1 ml 0.1 mol l^{-1} HCl. Samples (100 µl) of plasma, bile and extracts of the subpharyngeal region, head kidney and kidney were deproteinised with 4 volumes of methanol and centrifuged at 4°C (4000 *g*, 15 min). The supernatants were acidified with 1 volume of 1 mol l^{-1} HCl and loaded on to the column. The samples were then eluted from the column with 2 × 1 ml volumes of 0.1 mol l^{-1} HCl to separate free iodide, 6 × 1 ml volumes of H_2O to separate water-soluble conjugated forms of iodothyronines, and 3 × 1 ml volumes of 1 mol l^{-1} NH_3/ethanol to separate native iodothyronines. The radioactivity of the collected fractions was measured in a γ-counter.

Statistics

All data are presented as mean values ± s.d. Differences between groups were assessed by one-way ANOVA and Tukey's *post hoc* test. Statistical significance was accepted at $P < 0.05$ (two-tailed) and probabilities are indicated by asterisks (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$) and plus signs (+ $P < 0.05$; ++ $P < 0.01$; +++ $P < 0.001$).

Results

Autoradiography

Autoradiography demonstrated ^{125}I in the subpharyngeal region of tilapia. The radioactivity we observed in the intestinal tract was most probably caused by drinking (Fig. 1A, B). The inhibitory effect of perchlorate (Fig. 1H) hints at the involvement of the sodium-iodide symporter in the accumulation of radioiodide in tilapia intestine. In carp, ^{125}I was evident in the kidney but not in the subpharyngeal region (Fig. 1C, D). Furthermore, the gall bladder of carp contained radioactivity (Fig. 1E, F) which is in contrast with tilapia. Exposure to perchlorate blocked iodide accumulation in the subpharyngeal region of tilapia (Fig. 1G, H) and carp kidney, although radioactivity was still present in carp gall bladder (Fig. 1I, J).

^{125}Iodide tissue distribution

We retrieved 97±14% of the nominal amount of injected radioactivity from the tissue extracts. Measured 2 h after injection, the plasma 125I radioactivity in tilapia was 719 (± 290) × 10^3 c.p.m. g^{-1}, which decreased to 151 (± 110) × 10^3 c.p.m. g^{-1} at 96 h. In carp these values were 1190 (± 230) × 10^3 c.p.m. g^{-1} and 22 (± 12) × 10^3 c.p.m. g^{-1}. The subpharyngeal area in tilapia maximally accumulated ^{125}I 31-fold over plasma levels at 96 h into the chase (Table 1). All tissues other than the subpharyngeal region showed a decrease in radioactivity during this period (Fig. 2A). In carp, kidney, head kidney and bile were the only compartments where radioactivity increased over time (Fig. 2B). In carp, kidney tissue was able to accumulate ^{125}I more than 500-fold relative to plasma radioactivity

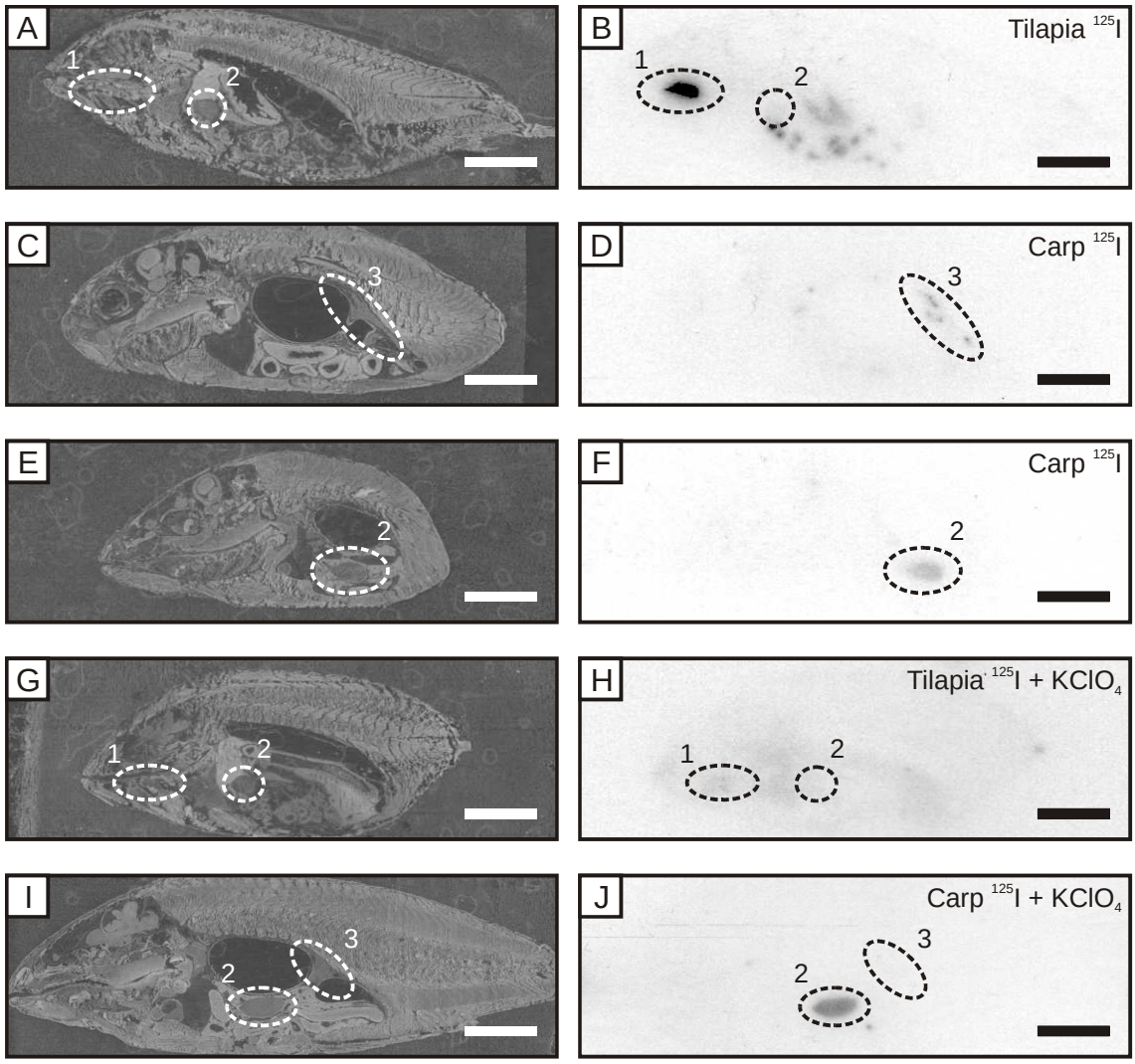

Figure 1. Representative autoradiographs of 30 μm, whole-body cryosections of juvenile tilapia (A, B) and juvenile carp (C-F) exposed to ^{125}I, and of tilapia (G, H) and carp (I, J) exposed to ^{125}I and $KClO_4$. Broken circles indicate the position of the subpharyngeal area (1), the gall bladder (2) and the kidney (3). Scale bars, 1 cm.

Table 1. Fold-increase in ^{125}I radioactivity relative to plasma ^{125}I radioactivity 96 h after i.p.-injection of radioiodide.

	Tilapia	Carp
Subpharyngeal area	31 ± 27	20 ± 13
Head kidney	0.5 ± 0.07	91 ± 54
Kidney	0.6 ± 0.05	544 ± 490
Bile	2 ± 3	37 ± 16

^{125}I radioactivity is given as Bq g^{-1} tissue (means ± s.d., *N*=6)

at 96 h. The head kidney and subpharyngeal area of carp also accumulated iodide, 91- and 20-fold, respectively, compared to plasma at 96 h (Table 1). While carp bile showed a 37-fold accumulation of ^{125}I at 96 h, tilapia bile radioactivity increased only twofold (Table 1).

Histology and morphological analysis

We could only detect thyroid follicles in the subpharyngeal area of tilapia, and this observation is corroborated by the ^{125}I tissue distribution shown in Fig. 2A. In carp, thyroid follicles were observed in kidney, subpharyngeal area and head kidney (Fig. 3A-C). The bright red stain of the lumen of follicular structures obtained with Crossmon's trichrome colocalized with a specific thyroxine-immunoreactivity, confirming that the follicles found in all three tissues are indeed thyroid follicles (Fig. 3D-F). On average, 1391 ± 196 (N = 4) follicle cross sections were observed in carp, of which 87 ± 2% was located within the kidney, 3 ± 1% within the head kidney, and 10 ± 2% within the subpharyngeal area.
Fig. 4 illustrates the non-Gaussian distribution of the frequency distribution of the area (Fig. 4A), the perimeter (Fig. 4B), the form factor (Fig. 4C), the roundness (Fig. 4D) and the aspect ratio (Fig. 4D) of thyroid follicles' colloids in the subpharyngeal area, head kidney and kidney. The modal value of the area of the kidney colloid is significantly smaller than that of the colloid in the subpharyngeal area and head kidney (Table 2). Also, the mode of the perimeter of the colloid is significantly smaller in the kidney follicles as compared to head kidney follicles (Table 2). Although the mode of the form factor of the colloid did not differ between the tissues, the mode of the roundness and aspect ratio did; the colloid in the subpharyngeal area was significantly rounder and less elongated than the colloid in the head kidney (Table 2). No differences (P = 0.192) were observed between the epithelial cell height of the subpharyngeal area (6.04 ± 0.12 μm, N = 4), head kidney (6.67 ± 0.68 μm, N = 4) and kidney follicles (6.54 ± 0.43 μm, N = 4).

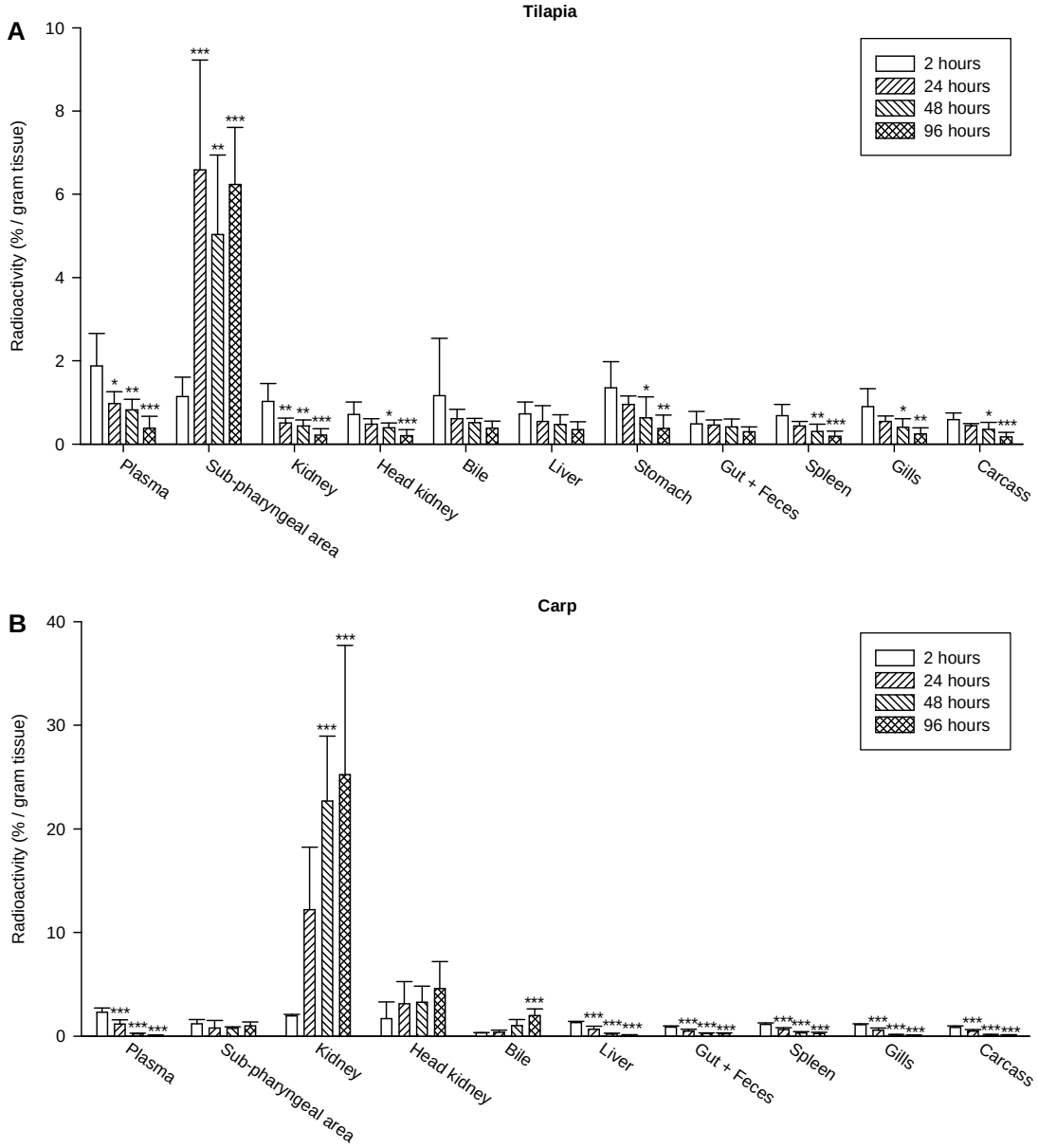

Figure 2. Tissue distribution of the radioactivity, shown as the percentage of the total dose injected in the fish per gram tissue at 2, 24, 48 and 96 h after i.p. ^{125}I injection in tilapia (A, N = 6) and carp (B, N = 6). Results are means ± s.d. One-way ANOVA was used for statistical evaluation. Asterisks show significant difference compared to the radioactivity level at 2 h (*P<0.05, **P<0.01, ***P<0.001).

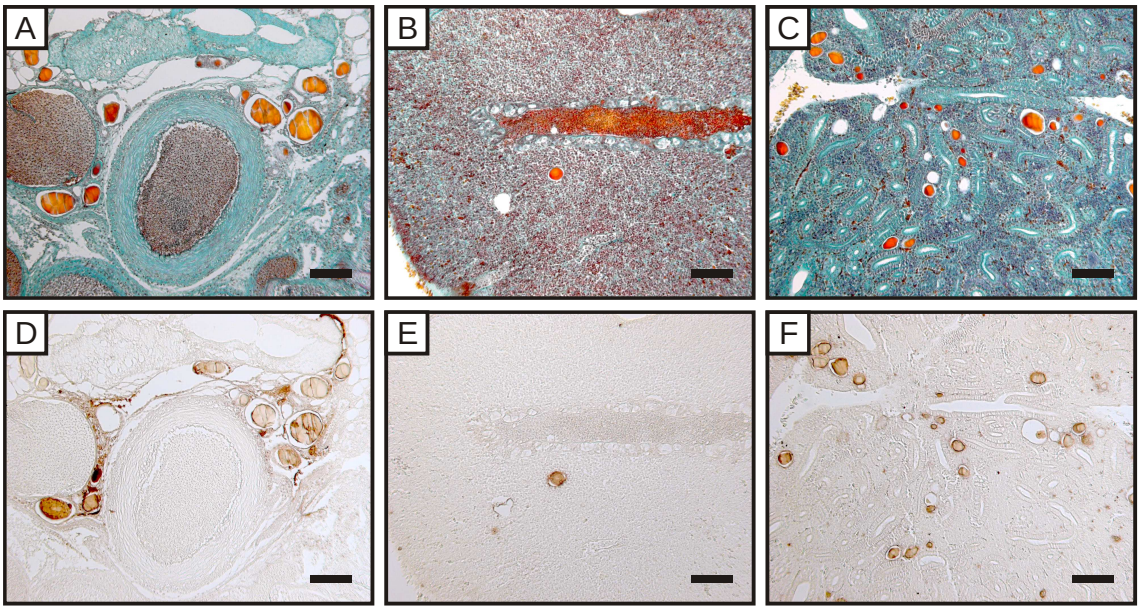

Figure 3. Crossmon's staining of 7 µm thick sections of carp subpharyngeal area (A), head kidney (B) and kidney (C). Thyroxine immunohistochemistry on serial sections of carp subpharyngeal area (D), head kidney (E) and kidney (F). Scale bars indicate 200 µm. For colour version of figure 3 see page 206.

Table 2. Mode of size and morphology descriptors for carp thyroid follicle colloid.

	Area (µm²)	Perimeter (µm)	Form Factor	Roundness	Aspect Ratio
Subpharyng. area	1403 ± 226[a]	151 ± 13[a,b]	0.80 ± 0.06[a]	0.75 ± 0.01[a]	1.18 ± 0.01[a]
Head kidney	1446 ± 283[a]	166 ± 30[a]	0.83 ± 0.05[a]	0.70 ± 0.02[b]	1.27 ± 0.03[b]
Kidney	963 ± 91[b]	125 ± 8[b]	0.88 ± 0.01[a]	0.72 ± 0.02[a,b]	1.23 ± 0.04[a,b]

One-way ANOVA was used for statistical evaluation. Different superscript letters indicate significant differences within the column, $P < 0.05$. Modes are shown ± s.d. ($N = 4$).

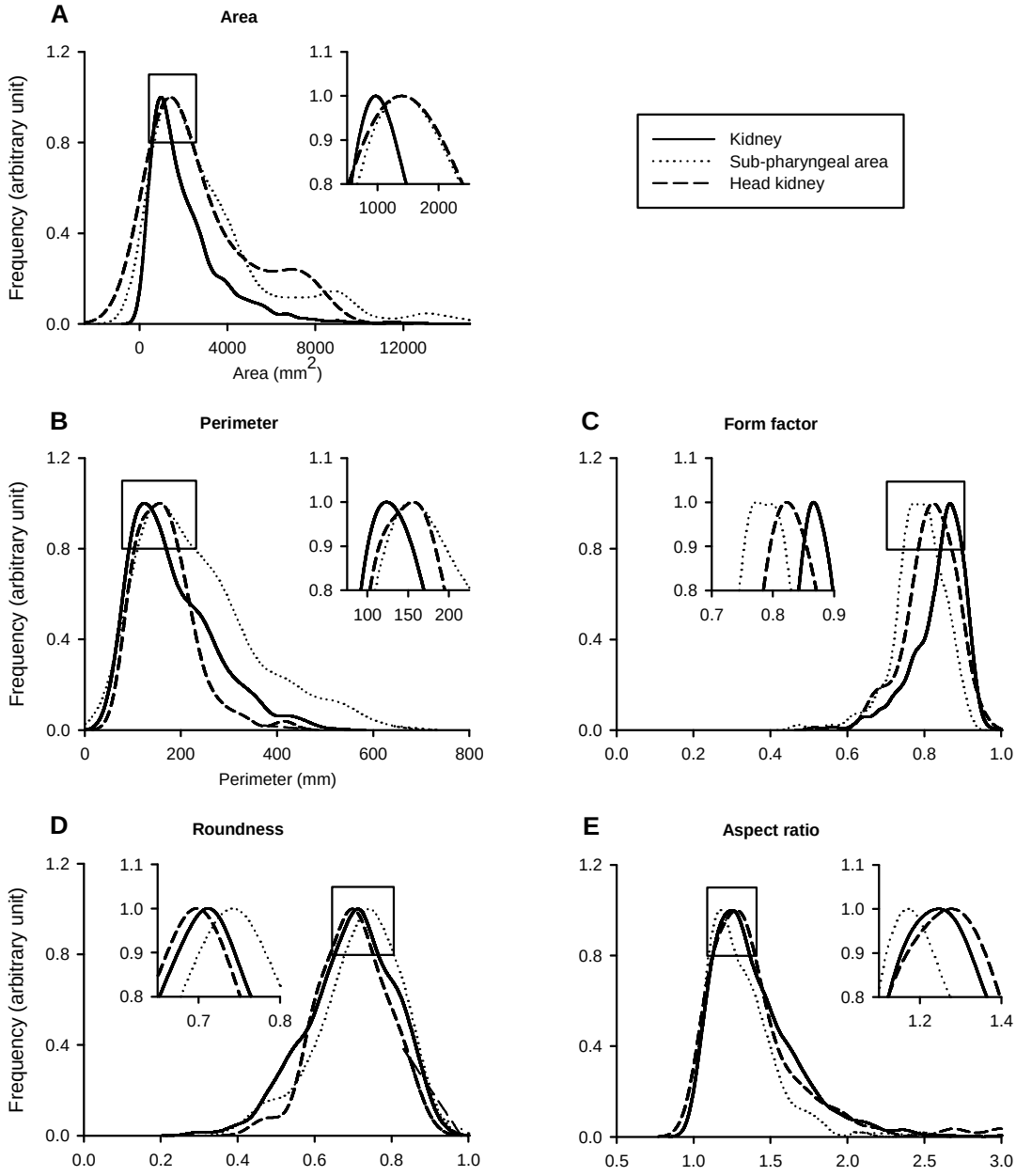

Figure 4. Frequency distributions of the area (A), perimeter (B), form factor (C), roundness (D) and aspect ratio (D) of the colloid of all individual thyroid follicles in a representative subpharyngeal area, head kidney and kidney of carp. Insert graphs represent the boxed area, to highlight the mode of the frequency distributions.

TSH-mediated T4 release in vitro

Exposure to 10 mIU bTSH/ml for 24 h significantly stimulated the release of T4 from carp kidney and head kidney tissue 1.7- and 3.6-fold, respectively (Fig. 5). Tissue from the subpharyngeal area was unresponsive to 10 mIU bTSH/ml.

^{125}Iodide pulse chase

Radio-labelled compounds were extracted with very high recoveries from the subpharyngeal area (92 ± 2% of total radioactivity), head kidney (77 ± 11%) and kidney tissue (92 ± 5%) in tilapia. Similar efficiencies were obtained for carp tissues (99 ± 1%, 96 ± 2%, and 96 ± 2%, respectively). In previous studies, extractions of radio-labelled thyroid hormones from tissues with ethanol or methanol combined with chloroform usually yielded recoveries of less than 70% and ranging from 45% to 94% (Crane, et al. 2004; Krysin 1990; Szisch, et al. 2005; Tagawa and Hirano 1987).

Chromatographic analysis of plasma revealed that the decrease in total radioactivity, as observed in Fig. 2, can mainly be attributed to a decrease of the tracer injected (^{125}I), which occurs at a faster rate in carp than in tilapia; between 2 h and 48 h after injection, ^{125}I plasma levels had decreased by 90 ± 6% in carp, whereas in tilapia, it had decreased by 48 ± 31% (Fig. 6). Radio-labelled, *i.e. de novo* synthesised, thyroid hormones appeared in increasing amounts in tilapia plasma during the experimental chase. In carp, however, newly synthesised thyroid hormones decreased from 2 h onwards. Conjugated forms of thyroid hormones appeared in tilapia plasma following the appearance of newly synthesised thyroid hormones.

The chronology of the appearance of labelled thyroid hormone metabolites in the subpharyngeal area, the head kidney and the kidney differed markedly between tilapia and carp. After an initial accumulation, iodide levels remained essentially constant as of 24 h in the subpharyngeal tissue of tilapia, whereas levels of labelled thyroid hormones and conjugates increased after 24 h (Fig. 7A). In kidney (Fig. 7B) and head kidney tissue (Fig. 7C) of tilapia no accumulation of iodide was observed. Small amounts of labelled thyroid

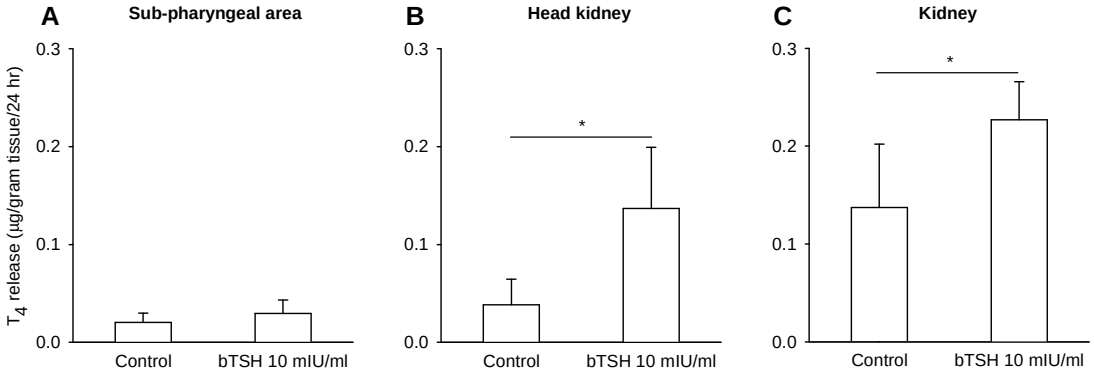

Figure 5. TSH (10 mIU ml^{-1})-mediated T4 release by carp subpharyngeal area (A, *N*= 7), head kidney (B, *N* = 7) and kidney (C, *N* = 7). Results are means ± s.d. **P*<0.05 (Student's *t*-test).

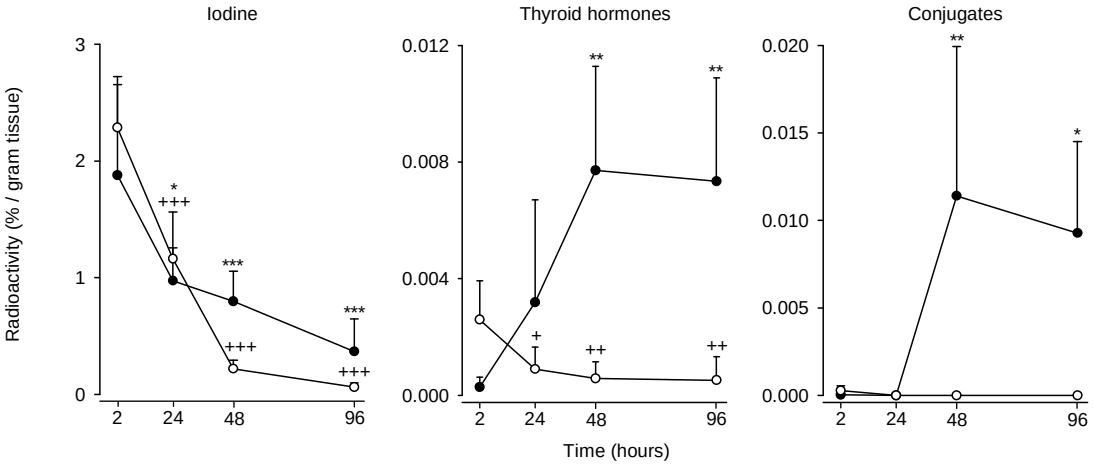

Figure 6. Radioactivity of iodide, thyroid hormone and thyroid hormone conjugates fractions, shown as the percentage of the total dose remaining in the fish at the time of sampling per gram of plasma, after Sephadex LH-20 chromatography at 2, 24, 48 and 96 h after i.p. ^{125}I injection in tilapia (●, *N* = 6) and carp (○, *n* = 6). Results are means ± s.d. One-way ANOVA was used for statistical evaluation. Significantly different levels of radioactivity compared with that at 2 h are indicated for tilapia (* *P* < 0.05, ** *P* < 0.01, *** *P* < 0.001) and carp (+ *P* < 0.05, ++ *P* < 0.01, +++ *P* < 0.001).

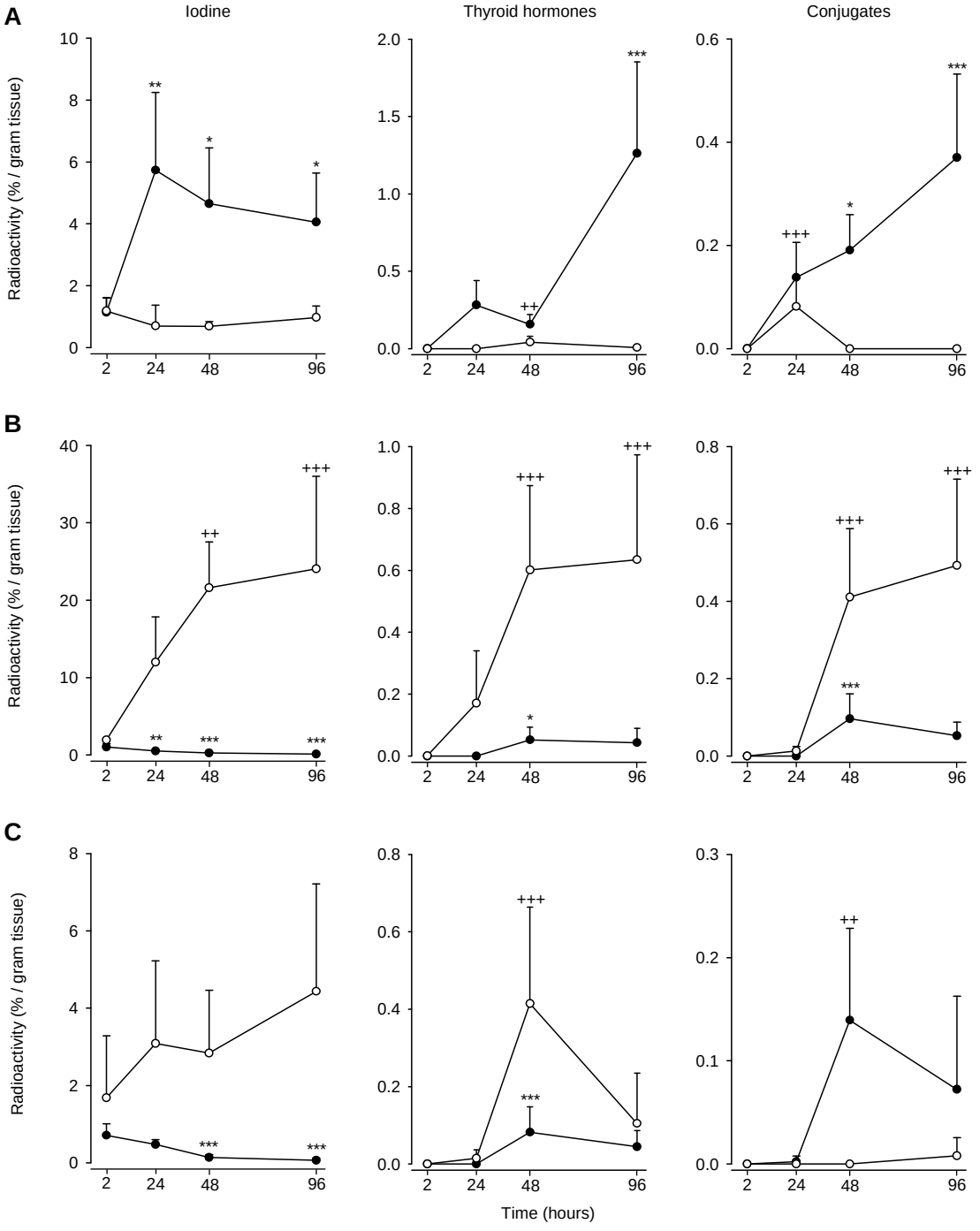

Figure 7. Radioactivity of iodide, thyroid hormone and thyroid hormone conjugates fractions, shown as the percentage of the total dose remaining in the fish at the time of sampling per gram tissue, in the subpharyngeal area (A), kidney (B) and head kidney (C), after Sephadex LH-20 chromatography at 2, 24, 48 and 96 h after i.p. ^{125}I injection in tilapia (●, $N = 6$) and carp (○, $N = 6$). Results are means ± s.d. One-way ANOVA was used for statistical evaluation. Significantly different levels of radioactivity compared with that at 2 h are indicated for tilapia (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) and carp (+ $P < 0.05$, ++ $P < 0.01$, +++ $P < 0.001$).

hormones and conjugates appear in these tissues at 48 h, which corresponds with the chronology seen in plasma, suggesting that these compounds originate from plasma.

In carp, maximum radioiodide accumulation in the kidney was reached at 48 h, after which the iodide level remained stable at 96 h (Fig. 7B). Thyroid hormones and thyroid hormone conjugates increased from 24 h onwards, reaching maximum levels at 48 h. Also in carp head kidney, newly synthesised thyroid hormones were observed as of 24 h, while thyroid hormone conjugates were essentially absent in this tissue (Fig. 7C). The subpharyngeal area of carp did not accumulate detectable radioiodide and virtually no radiolabelled thyroid hormones and thyroid hormone conjugates were observed (Fig. 7A).
Chromatographic analysis of bile revealed an increase in radioiodide content in carp bile, whereas in tilapia bile, radioiodide levels did not change significantly (Fig. 8A). Thyroid hormones and conjugated forms of thyroid hormone accumulated in bile of both species. The average total volume of the bile of both fish species did not decrease during the experiment, indicating that the gall bladder had not emptied in the intestinal tract during the chase period of the experiment.

In the ambient water of both fish, iodide, thyroid hormones and thyroid hormone conjugates were observed (Fig. 8B). Equal amounts of iodide and thyroid hormones were excreted by the two fish species. Conjugated thyroid hormones, however, were only excreted by tilapia.

Discussion

We observed remarkable differences in the location of active endocrine thyroid tissue between carp and tilapia. In carp three thyroid follicle populations, active and inactive, were identified in subpharyngeal area, head kidney and kidney. Also, differences at the level of the peripheral metabolism of thyroid hormones were observed. Not only did plasma clearance differ, but also the route of thyroid hormone excretion varied between carp and tilapia.

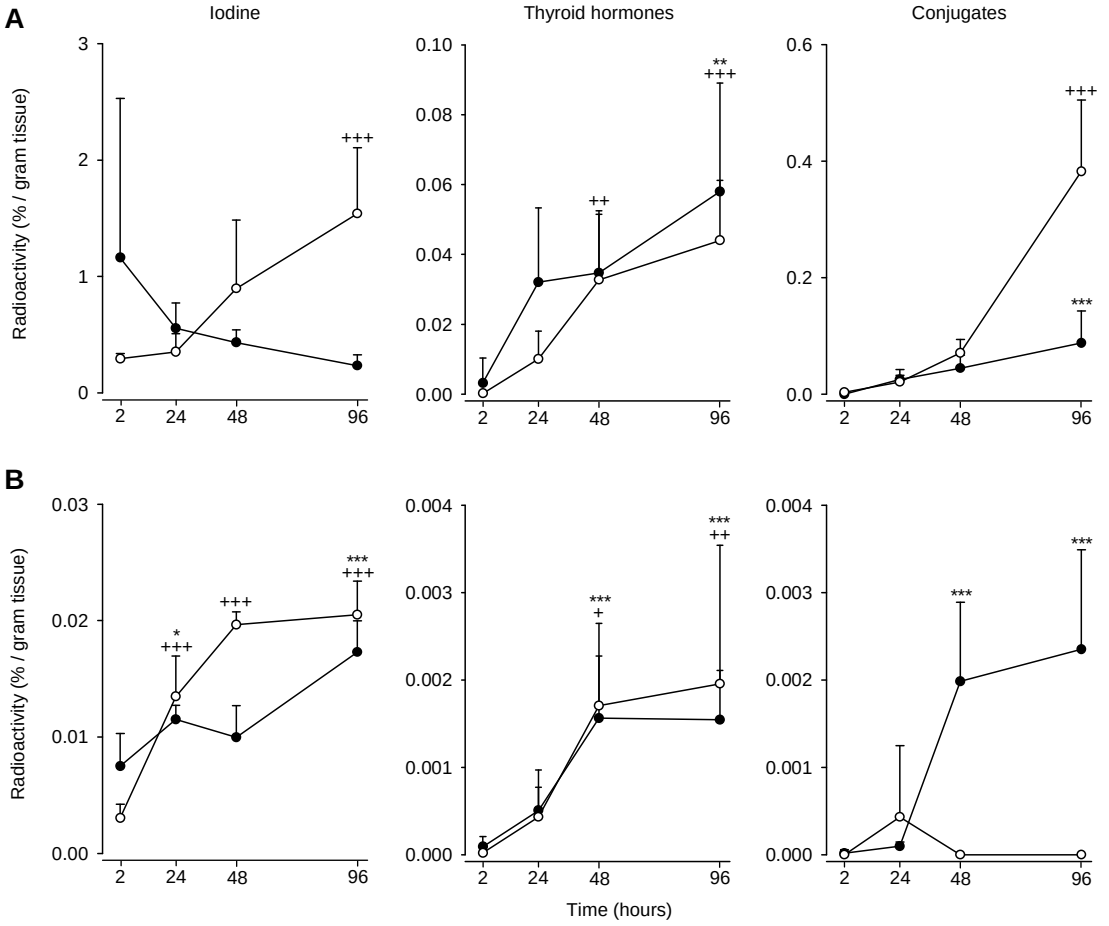

Figure 8. Radioactivity of iodide, thyroid hormone and thyroid hormone conjugates fractions, shown as the percentage of the total dose remaining in the fish at the time of sampling per gram tissue, in the bile (A) and the ambient water (B), following Sephadex LH-20 chromatography at 2, 24, 48 and 96 h after i.p. ^{125}I injection in tilapia (●, n = 6) and carp (○, n = 6). Results are menas ± s.d. One-way ANOVA was used for statistical evaluation. Significantly different levels of radioactivity compared with that at 2 h are indicated for tilapia (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) and carp (+ $P < 0.05$, ++ $P < 0.01$, +++ $P < 0.001$).

In tilapia the subpharyngeal area was the only site in which thyroid follicles were found, where perchlorate-sensitive iodide accumulation was observed, and where thyroid hormones were synthesised *de novo*. This demonstrates, for tilapia, a location and activity typical for the teleostean thyroid gland. The anatomical location of the thyroid gland in carp, however, deviates from that in tilapia. In carp, the renal tissues display thyroid activity as evidenced by iodide accumulation, confirming the observations of Leray and Febvre (1968) and Lysak (1964). Although both head kidney and kidney in carp

synthesised thyroid hormones and secreted thyroid hormones following TSH stimulation, the head kidney can have only a moderate share in total thyroid output. Not only was the kidney the foremost iodide-accumulating tissue in carp, which was inhibited by perchlorate, it also harbours the largest amount of thyroid tissue: 87% of the total thyroid follicle population, as opposed to 3% in the head kidney. This suggests a significant role for the kidney thyroid follicles in thyroid economy of carp.

The most striking aspect of this study is the absence of iodide accumulation and thyroid hormone synthesis in the subpharyngeal region of carp, despite the presence of thyroid follicles. Furthermore, we found that carp subpharyngeal thyroid follicles do not have the capacity to release thyroid hormones upon stimulation with TSH *in vitro*, whereas renal thyroid follicles do. This, together with the high prevalence, establishes the kidney as the anatomical site of the thyroid gland in this species. These results may pose questions whether to (re-)consider the term 'heterotopic' in conjunction with 'thyroid follicles' in common carp. In goldfish (*Carassius auratus*), a species closely related to the common carp, the subpharyngeal thyroid follicles are active and are responsible for 11-40 % of total iodide accumulation (Chavin and Bouwman 1965; Peter 1970), leaving a considerable role for subpharyngeal thyroid follicles in the uptake of iodide in this species. However, the subpharyngeal follicles in goldfish appear not to be responsive to T4 treatment; changes in thyroid activity, *i.e*. iodide accumulation and epithelial cell height, are primarily mediated through inter-renal thyroid follicles, which shows that the thyroid populations are not physiologically equivalent (Peter 1970).

Histologically, the subpharyngeal follicles in carp appear normal, active and similar to kidney thyroid follicles, as evidenced by the epithelial cell height, which does not differ significantly from that of kidney follicles. Differences in size and shape were observed between the thyroid follicle populations, *viz*. the colloid of kidney follicles is the smallest, and subpharyngeal follicles appear to be more round than renal follicles. It is tempting to interpret the small colloidal area of renal thyroid follicles as an indication of increased colloidal resorption, which, again, is indicative of increased hormonogenesis in these follicles. The latter is corroborated by our observation of increased iodide accumulation and the presence of *de novo* synthesised thyroid hormones in this region.

Studies from Raine and colleagues (Raine and Leatherland 2000; Raine, et al. 2005) show that in embryonic rainbow trout (*Oncorhynchus mykiss*) the functional unit of the thyroid gland appears tubular, not follicular, and that this morphology is retained in the juvenile stages. Despite the fact that carp thyroid tissues were sectioned in varying planes, we did not detect tubular structures in our serial sections. A significant proportion of tubular colloid-filled structures would also have been made apparent by a large variation in the morphology descriptors of the thyroidal colloid. Instead, they show little variation. Of course, it remains to be determined whether measurement of thyroidal colloid accurately reflects thyroid follicle morphology. Still, one can speculate that differences in morphometrics might reflect differences in organogenesis between species.

Although the subpharyngeal thyroid follicles in carp did not incorporate radioiodide to a significant degree or synthesise thyroid hormones *de novo*, these subpharyngeal follicles do show T4 immunoreactivity. Apparently these follicles do have an intrinsic capacity to synthesise thyroid hormones. Whether the rate of thyroid hormone synthesis is to slow to detect within the 96 h of the experimental chase, or whether these follicles were active during earlier life stages and are now dormant (and still contain T4) remains to be determined. Interestingly, neotenic urodeles are able to complete a full life cycle without metamorphosis (Rosenkilde and Ussing 1996). Because of an impaired thyroid system these amphibians are unable to release a surge of thyroxine, necessary to initiate metamorphosis. Although intact and functional, their thyroid system is relatively inactive at several levels of the thyroid axis, from the central regulation of the thyroid gland to the peripheral deiodination of thyroid hormones. Neoteny is also described in fish; during adult life the ice goby (*Leucopsarion petersii*) exhibits several larval characteristics, indicative of an impaired metamorphosis. During its development, the thyroid follicles are smaller and have a lower epithelial cell height when compared with a metamorphic goby species; also no TSH immunoreactivity was observed in the pituitary (Harada, et al. 2003). Although carp are not neotenic, further research on the carp subpharyngeal thyroid follicles may provide more insight in the mechanisms controlling the non-pharmacologically induced inactivity of the thyroid gland as observed in neotenic organisms. A possible mechanism could

be the temporal expression of active and/or inactive splice variants of key-regulators of thyroid hormone synthesis, e.g. TSH-receptor, sodium-iodide symporter or thyroglobulin.

It is unclear why the functional endocrine thyroid tissue is located in the kidney and not in the subpharyngeal area. We hypothesise that two, potentially functional, thyroid populations with different sensitivities to thyrotropic factors, or with different synthesising properties, confer an accurate regulation of thyroid gland output in response to a demand for systemic thyroid hormone. It can also be hypothesised, regarding the close juxtaposition of the extra-pharyngeal thyroid follicles to specific cell-types in the head kidney and kidney, that thyroid hormones have a paracrine effect on inter-renal (cortisol-producing), chromaffin (catecholamine-producing), and/or haematopoietic cells or on nephron structures. Paracrine relationships between the stress axis and immune system have already been demonstrated in the multifunctional carp head kidney (Metz, et al. 2006). Attempts to demonstrate a direct *in-vitro* effect of thyroid hormones on the release of cortisol in carp head kidney have not been successful yet, even though treatment of carp with thyroxine resulted in a decrease in the levelsof plasma cortisol (Geven, et al. 2006).

The presence of ^{125}I radioactivity in carp bile and its absence in tilapia bile suggests a faster turnover rate of thyroid hormones in carp than in tilapia. This is corroborated by the faster clearance of iodide and thyroid hormones from plasma, and the accumulation of iodide and thyroid hormone conjugates in bile of carp compared to tilapia. Not considering differences between species, these results appear to contradict the general idea that higher temperatures will result in increased thyroid activity (Eales, et al. 1982), as our carp are held at a temperature that is 5°C lower than that of tilapia.

The appearance of thyroid hormone conjugates in the plasma of tilapia is consistent with the observations of DiStefano, et al. (1998), who found a significant amount of T3 glucuronides in plasma of Mozambique tilapia after i.p. injection with [^{125}I]T3 ([^{125}I]3,5,3'-triiodothyronine). Although thyroid hormone sulfates are found in the sera of several mammals (Santini, et al. 1993; Wu, et al. 1992; Wu, et al. 1993), and indirect evidence exists for thyroid hormone conjugates in plasma of European plaice (*Pleuronectes platessa* L.) (Osborn and Simpson 1969), the Mozambique tilapia appears to be the only

vertebrate in which plasma thyroid hormone glucuronides are observed. By injection of trace amounts of radioiodide instead of radiolabelled thyroid hormones, we circumvented the possibility of altering the thyroid status of the fish. The injection of radioiodide, as opposed to radiolabelled thyroid hormones, also allowed us to speculate on the anatomical site at which conjugated thyroid hormones are produced. Whereas thyroid hormone conjugates were observed in tilapia plasma at 48 and 96 h after i.p. injection, they were already present in the subpharyngeal area at 24 h, suggesting that the glandular thyroid gland itself may be responsible for the production of thyroid hormone conjugates in tilapia plasma. In this respect, the finding of conjugated forms of thyroid hormones in the kidney of common carp, which harbours the functional thyroid, is remarkable. However, we cannot exclude the possibility that cell types other than thyroid tissue, are responsible for the presence of thyroid hormone conjugates.

The thyroid hormone conjugates in tilapia plasma have been suggested to function as a pool of thyroid hormones from which, by deconjugation, a rapid mobilisation of bioactive thyroid hormones is available (DiStefano, et al. 1998). Our results, however, suggest that the thyroid hormone conjugates in tilapia plasma are involved in the excretion of thyroid hormones, through routes other than bile. The appearance of thyroid hormone conjugates in the ambient water coincides with the appearance of thyroid hormone conjugates in the plasma, suggesting that thyroid hormone conjugates are excreted via plasma, possibly through the gill or kidney. Since the volume of the gall bladders did not decrease during the experiment and leakage of thyroid hormone conjugates over the gall bladder wall in fish is negligible (Collicutt and Eales 1974), the thyroid hormone conjugates in the ambient water are unlikely to originate from the bile. It is striking that in channel catfish (*Ictalurus punctatus*), 8.1 % of all injected [^{125}I]T4 is excreted via routes other than the gall bladder (Collicutt and Eales 1974) and that in rainbow trout (*Oncorhynchus mykiss*), 8.2 % and 6.7 % of injected [^{125}I]T4 and [^{125}I]T3, respectively, was excreted via urine (Parry, et al. 1994). These reported percentages are of the same order of magnitude as the observed percentage (8.3%) of thyroid hormone conjugates found in tilapia plasma, supporting the idea that plasma thyroid

hormone conjugates in tilapia may function in the excretion of thyroid hormone metabolites (DiStefano, et al. 1998).

In summary, we have shown that thyroid hormone synthesis, anatomical location and activity of thyroid tissue, and thyroid hormone excretion in teleost fish differ greatly between two species. The most distinct feature of teleost thyroid physiology observed in this study is the presence of a completely functional endocrine thyroid gland in the renal tissues of common carp. This finding may open new possibilities for *in vitro* studies on fish thyroid.

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Chapter 3

Histological analysis of the ontogeny of thyroid tissue in early life stages of common carp (*Cyprinus carpio* L.)

Edwin J. W. Geven, Auke Widow, Gert Flik and Peter H. M. Klaren

Abstract

In juvenile common carp (*Cyprinus carpio* L.) thyroid follicles are not only located in the typical subpharyngeal region, they are also observed in the head kidney and kidney. Functional studies revealed that only the renal thyroid follicles are active, although the subpharyngeal thyroid follicles did exhibit thyroxine-immunoreactivity (T4-ir), and this suggests that these follicles were once active, possibly during early life stages. To address this, we investigated the ontogeny of thyroid tissue in common carp during the first 36 days post hatching (dph), using (immuno-)histochemical techniques. T4-ir was only observed in the subpharyngeal region and not in the renal tissues, suggesting that the subpharyngeal thyroid is indeed functional and that renal thyroid follicles are not involved in major developmental processes. The T4-ir in the first month represented intracellular thyroid hormone synthesis, as the first thyroid follicles were only identified at 32 dph. This deviates from the general idea that thyroid hormone synthesis is dependent on a follicular structure. A biochemical basis for such extracellular thyroid hormone synthesis is discussed as well as its physiological relevance. We postulate that intracellular thyroid hormone synthesis is a physiological relevant thyroid hormone retrieval strategy, which is employed by common carp, next to the retrieval of maternal thyroid hormones from yolk and follicular thyroid hormone synthesis at a juvenile and adult stage.

Introduction

Thyroid hormones are synthesised in thyroid follicles, which form the functional units of the thyroid gland. A thyroid follicle is composed of a monolayer of thyrocytes, which encloses a protein-filled extracellular matrix, the colloid, of which thyroglobulin is the main constituent. The thyroid gland is the only endocrine gland that synthesises and stores its hormone product extracellularly, and the three-dimensional structure of the thyroid follicle reflects just that. Indeed, crucial steps in thyroid hormone biosynthesis such as the oxidation of iodide to the reactive species iodonium by thyroid peroxidase, and its incorporation into thyroglobulin's tyrosine residues to form iodothyronines, all occur in the extracellular colloid in the follicular lumen.

The development of the thyroid gland in fish largely resembles that in mammals. Briefly, endodermal cells from the thyroid anlage, situated in the midline of the embryonic pharyngeal floor, are determined to develop into thyroid cells. After proliferation of the thyroid anlage, primordial cells migrate to their ultimate anatomical location, *viz*. the ventral neck region in mammals and the subpharyngeal region in teleostean fish. Upon arrival the primordial cells differentiate and organise into functional thyroid follicles. In fish, the thyroid follicles then multiply and migrate caudally along the anterior-posterior axis of the ventral aorta (Alt, et al. 2006; De Felice and Di Lauro 2004; Elsalini, et al. 2003; Wendl, et al. 2002).

Whereas in mammals and other vertebrates the thyroid gland is a compact and encapsulated gland, in teleost fish and adult cyclostomes the thyroid gland is a loose organisation of non-encapsulated thyroid follicles scattered in the subpharyngeal region along the ventral aorta (Capen 2000; Gudernatsch 1911). The lack of a capsule may explain the occurrence of thyroid follicles in tissues and organs other than the subpharyngeal region in several species of fish. These so-called heterotopic, or "misplaced", thyroid follicles have been observed in a variety of organs, *e.g.* heart, spleen, liver, oesophagus, brain and choroid rete mirabile, but in particular in renal tissues, the head kidney and kidney (Baker 1958). Heterotopic thyroid follicles have been described in species throughout the Teleostei infraclass, half of which belong to the family of carp and minnows (Cyprinidae), including common carp (*Cyprinus carpio* L.) and goldfish (*Carassius auratus*) (Geven, et al. 2007).

Heterotopic thyroid follicles are functional and are therefore assumed to work in concert with the subpharyngeal thyroid tissue to maintain the organism's thyroid status (Bhattacharya, et al. 1976; Chavin and Bouwman 1965; Frisen and Frisen 1967; Peter 1970; Srivastava and Sathyanesan 1971).

In adult common carp, thyroid follicles are found in the subpharyngeal region, the head kidney and kidney. When we injected carp with radioiodine (^{125}I), and incubated tissues *in vitro* with thyroid-stimulating hormone (TSH) we found that kidney and head kidney actively accumulated iodine, and synthesised and secreted thyroid hormones, whereas the subpharyngeal region did not (Geven, et al. 2007). Immunohistochemical analysis revealed thyroxine-immunoreactivity (T4-ir) in the luminal colloid of all thyroid follicles, including those in the subpharyngeal region, suggesting that the subpharyngeal thyroid follicles were once, during earlier life stages, capable of hormonogenesis but are now dormant. Alternatively, iodide turnover is too slow, or thyroid hormone synthesis is too low to detect in these dormant follicles.

We hypothesise that during early ontogeny in common carp, first the subpharyngeal thyroid is developed and is active, whereupon at a later time-point the renal thyroid emerges and the subpharyngeal thyroid becomes inactive. Information on the developmental stage at which heterotopic thyroid follicles appear may provide further insight in the physiological relevance of thyroid heterotopia. We therefore investigated the early ontogeny of the thyroid gland in common carp larvae during the first 36 days after hatching, using histochemical and immunohistochemical techniques.

Materials and methods

Fertilization and incubation of eggs

Carp eggs and sperm were obtained from the Department of Fish Culture and Fisheries at Wageningen University (The Netherlands). The gametes were mixed, and *in vitro* fertilisation was induced by the addition of city of Nijmegen tap water. Non-fertilised eggs were removed, and eggs were incubated at a temperature of 23°C in 10-l tanks with circulating aerated tap water. Eggs hatched at the third day after fertilisation, conform standard carp development

where hatching takes place around 55 hours post-fertilisation (Oyen, et al. 1991). After yolk resorption the larvae were fed live *Artemia sp.* nauplii.

Histochemistry

Every fourth day for a period of 36 days after hatching, five larvae were sampled. Larvae were killed by immersion in 0.1% (v/v) 2-phenoxyethanol and were fixed *in toto* in Bouin's solution for 24 h. Fixed specimens were dehydrated in a graded series of ethanol, embedded in paraplast and sectioned at 7 μm thickness. Serial sections were collected and mounted on glass slides. A modified Crossmon's connective tissue stain, followed by a haematoxylin counter-stain (Geven, et al. 2007) was employed. This procedure stains the colloid of thyroid follicles in adult carp bright orange-red. Kidney from adult carp served as a positive control for the staining procedure.

Immunohistochemistry

Adjacent sections from the sections used for histochemistry were examined for thyroxine-immunoreactivity (T4-ir). In short, the sections were incubated with 2% H_2O_2 and 10% normal goat serum in ice-cold phosphate-buffer to inactivate endogenous peroxidase activity and to block non-specific antigenic sites. Sections were then incubated overnight with a polyclonal antiserum against thyroxine (MP Biomedicals, Irvine, CA, USA) at a dilution of 1:5,000. Then, sections were incubated for 1 hour with 1:200 biotinylated goat anti-rabbit secondary antibody (VectaStain, Vector Laboratories, Burlingame, CA, USA) and incubated for 30 min with VectaStain ABC reagent. Antibody binding was detected using 0.025% 3,3'-diaminobenzidine (Sigma Chemical Co., St. Louis, MO, USA) in the presence of 0.02% H_2O_2. Again, sections of kidney from adult carp were included as a positive control.

Results

Successful staining procedures were confirmed in kidney tissue of adult carp, where thyroid colloid stained brightly red following Crossmon's staining, and the thyroid follicles displayed T4-ir. Analysis of larval head kidney and kidney stained with Crossmon's stain or thyroxine-immunohistochemistry did not reveal any follicular structure or T4-ir, respectively. Also, examination of the larval subpharyngeal region following Crossmon's staining did not reveal any apparent red stained follicular structures (Fig. 1A, C, E).

Pronounced T4-ir was observed in the sub-pharyngeal region of larvae of all ages examined (Fig. 1B, D, F). At an age of 4 dph two out of five larvae exhibited T4-ir in the subpharyngeal region, whereas from 8 dph onwards all larvae showed T4-ir, except at 20 dph where three out of the five fish revealed T4-ir. Examination of the adjacent Crossmon's stained sections learned that the T4-ir was not located in the lumen of follicular structures, but intracellularly in individual cells or groups of cells (Fig. 1A, B, C, D).

The first time point that a follicular structure, *viz*. thyrocytes surrounding a colloid, was observed was at 32 dph (Fig. 1E, F). At an age of 32 and 36 dph the colloid was stained slightly pink, which is distinctly lighter than the bright red staining in adult fish. At 32 and 36 dph, respectively, 9.7 ± 8.3 (s.d.) and 6.9 ± 1.7 % of total T4-ir was detected in follicular structures.

Discussion

We here show that the thyroid follicle populations in the subpharyngeal region, head kidney and kidney of common carp not only differ functionally (Geven, et al. 2007), but also temporally, as they do not develop simultaneously.

While subpharyngeal thyroid follicles are present during the first month of post-hatch development, renal thyroid follicles did not appear in this period. We have previously reported the presence of functional renal thyroid follicles in 8-months old juveniles (Geven, et al. 2007). Clearly, renal thyroid follicles are not involved in the regulation of major developmental processes in the first month after hatching.

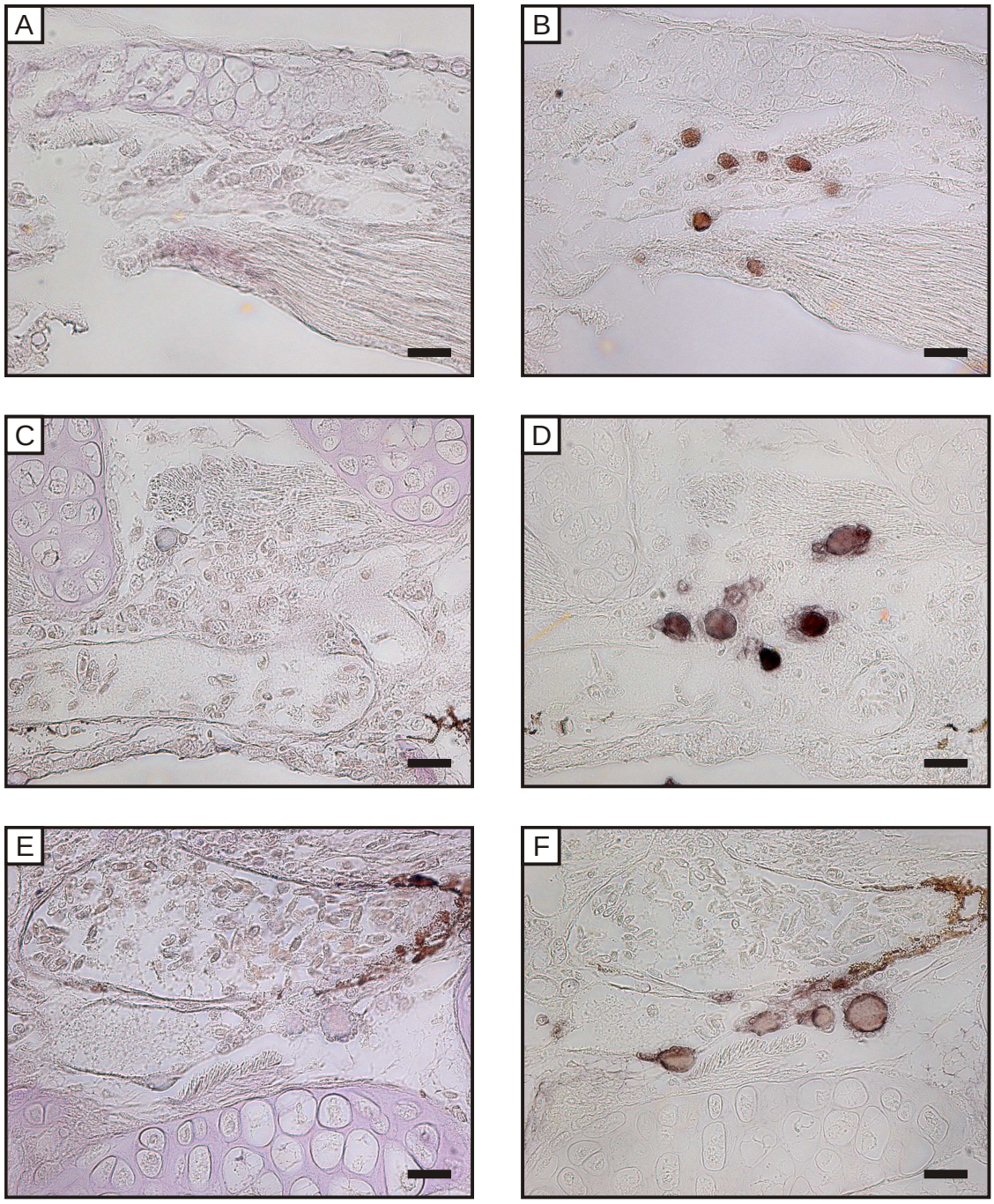

Figure 1. Crossmon's staining of 7 μm sections of the subpharyngeal area of carp at 8 (A), 20 (C) and 32 dph (E). Thyroxine immunoreactivity in adjacent sections of the subpharyngeal area of carp at 8 (B), 20 (D) and 32 dph (F). Scale bars indicate 20 μm. For colour version of figure 1 see page 207.

Before the development of thyroid tissue, fish larvae are dependent on maternally derived thyroid hormones which are stored in the yolk (Power, et al. 2001; Tagawa and Hirano 1990). In teleost fish, endogenous thyroid hormone synthesis is initiated before, or directly after, yolk resorption (Einarsdóttir, et al. 2006; Klaren, et al. 2008; Nacario 1983; Raine and Leatherland 2000; Wabuke-Bunoti and Firling 1983; Wendl, et al. 2002). This will ensure adequate systemic thyroid hormone levels for the orchestration of future developmental processes. Since in common carp the yolk is fully resorbed within 4 dph, we are confident that the subpharyngeal T4-ir during the first month after hatching represents endogenously synthesised thyroid hormones.

The finding of evidence for intracellular thyroid hormone synthesis was surprising since thyroid hormone synthesis is generally believed to depend on the three-dimensional structure of the thyroid follicle, which facilitates the extracellular synthesis and storage of iodothyronines. Indeed, in rainbow trout (*Oncorhynchus mykiss*) and Senegalese sole (*Solea senegalensis*) the first T4 -ir is associated to the lumen of follicle structures (Klaren, et al. 2008; Raine and Leatherland 2000). The non-follicular thyroid cells in common carp are located in the exact anatomical location as the to be formed thyroid follicles, suggesting that these cells are indeed the progenitors of the future thyroid follicles.

Examples of intracellular thyroid hormone synthesis are found *in vitro* as well as *in vivo*. Although devoid of an extracellular colloid, various mammalian thyrocytes monolayer culture systems do exhibit thyroid hormone biosynthesis and secretion, albeit at a very low rate compared to intact thyroid follicles (Kimura, et al. 2001; Kuliawat and Arvan 1994; Roger, et al. 1997). *In vivo*, non-follicular cells in the endostyle of members of the subphyla Cephalochordata (amphioxus) and Urochordata (ascidians) and of non-parasitic larvae of lamprey and hagfish are responsible for the synthesis of thyroid hormones (Eales 1997). These examples illustrate that non-follicular intracellular thyroid hormone synthesis is feasible and that it is biologically relevant in a number of species.

In thyroid follicles, thyroid hormones are synthesised in the extracellular matrix, the colloid, which sets a specific biochemical environment for the iodination of thyroglobulin by thyroid peroxidase. The extracellular matrix is formed either by endosomes that fuse with an already established intercellular

cavity or by intracellular cavities that fuse and following cell division will form the colloid (Toda, et al. 2001). Whereas in mammalian pre-follicular thyrocytes thyroglobulin is not yet expressed (Toda, et al. 1993), in common carp it appears that thyroglobulin gene expression is not dependent on thyroid follicle formation. This allows for intracellular thyroid hormone synthesis in either endosomes or intracellular cavities, after which thyroid hormones can be secreted via regular endocytotic pathways.

Next to follicular- and intracellular thyroid hormone synthesis, another thyroid hormone retrieval strategy has been described. It has been suggested that several aquatic invertebrates take up thyroid hormones from an exogenous source, *e.g.* food (Heyland and Moroz 2005). Uptake of exogenous thyroid hormones, either from food or yolk, is a low-cost strategy to obtain thyroid hormones since it bypasses the energy-dependent uptake of iodide and a synthesising machinery. However, it is fully dependent on the availability on exogenous sources of thyroid hormones, which makes this strategy rather opportunistic. Since thyroid follicles are highly efficient in iodine uptake and are able to store thyroid hormones, this strategy is relatively independent of exogenous iodine levels, albeit with higher metabolic costs to invest. Cellular thyroid hormone synthesis may represent an intermediate strategy. Although it is not dependent on exogenous thyroid hormones, it still requires a continuous supply of iodine as it is not able to store thyroid hormones extracellularly. Interestingly, intracellular thyroid hormone synthesis has thus far only been observed in aquatic species, *viz.* amphioxus, ascidians, lamprey, hagfish and teleost fish, where iodine is readily available. Since intracellular thyroid hormone synthesis requires only a singe endocytotic pathway, which resembles steroid hormone producing cells, the secretion of thyroid hormones is relatively quick when compared to follicular thyroid hormone synthesis. This may be advantageous during early ontogeny when a quick supply of thyroid hormones is required for the regulation of the developmental processes. Indeed intracellular thyroid hormone synthesis is only observed during the early development of fish and in larval lamprey and hagfish.

Within this context is it remarkable to find all these three strategies at different life stages of common carp; *viz.* the retrieval of maternal thyroid hormones from the yolk, followed by intercellular thyroid hormone synthesis

and finally extracellular thyroid hormone synthesis. Therefore, further investigations on thyroid hormone synthesis in teleost fish may provide new insights on the evolution of thyroid hormone handling in vertebrates.

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Chapter 4

Experimental hyperthyroidism and central mediators of stress axis and thyroid axis activity in common carp (*Cyprinus carpio* L.)

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Abstract

The effect of experimental hyperthyroidism, realised by T4 injection, on central mediators of the hypothalamo-pituitary-interrenal axis (HPI-axis) in common carp (*Cyprinus carpio* L.) was studied. Our results show that hyperthyroidism evokes a marked 3.2-fold reduction in basal plasma cortisol levels. Corticotropin-releasing hormone-binding protein (CRH-BP) mRNA levels in the hypothalamus, measured by real-time quantitative PCR, were significantly elevated by 40%, but CRH, urotensin-I, prepro-TRH, prohormone convertase-1 (PC1) and POMC mRNA levels were unchanged. In the pituitary pars distalis, PC1, CRH receptor-1 and POMC mRNA levels were unaffected, as was ACTH content. Plasma α-MSH concentrations were significantly elevated by 30% in hyperthyroid fish, and this was reflected in PC1 and POMC mRNA levels in pituitary pars intermedia that were increased 1.5- and 2.4-fold, respectively. The α-MSH content of the pars intermedia was unchanged. Hyperthyroidism has profound effects on the basal levels of a central mediator, *i.e.* CRH-BP, of HPI-axis function in unstressed carp *in vivo*, and we conclude that HPI- and hypothalamo-pituitary-thyroid-axis functions are strongly interrelated. We suggest that the changes in plasma cortisol, thyroid hormone, and α-MSH levels reflect their concerted actions on energy metabolism.

Introduction

The synthesis and secretion of thyroxine (T4) by the thyroid gland are stimulated by thyroid-stimulating hormone (TSH) from the pituitary gland. In mammals, TSH secretion is under positive hypothalamic control by thyrotropin-releasing hormone (TRH), and thyroid hormones exert a classical negative feedback on the expression of TSH (Shupnik 2000) and TRH (Guissouma, et al. 1998; Kakucska, et al. 1992; Koller, et al. 1987). In teleosts also, inhibitory regulation by thyroid hormones of pituitary TSH expression has been confirmed (Chatterjee, et al. 2001; Larsen, et al. 1997; Pradet-Balade, et al. 1997; Yoshiura, et al. 1999), but, to the best of our knowledge, the only non-mammalian study showing an inhibitory effect of T3 and T4 on the expression of TRH was conducted on chicken (Lezoualc'h, et al. 1992).

Studies on bighead carp (*Aristichtys nobilis)*, arctic charr (*Salvelinus alpinus*) and Japanese eel (*Anguilla japonica*) indicate a role for TRH in fishes similar to that in mammals (Chatterjee, et al. 2001; Eales and Himick 1988; Han, et al. 2004). However, in the lungfish (*Protopterus ethiopicus*), Nile tilapia (*Oreochromis niloticus*) and common carp (*Cyprinus carpio*) TRH does not stimulate TSH secretion (Gorbman and Hyder 1973; Kagabu, et al. 1998; Melamed, et al. 1995). In several non-mammalian vertebrates, including teleosts, factors other than TRH were found to be thyrotropin-releasing factors. Indeed, in coho salmon (*Oncorhynchus kisutch*), corticotropin releasing hormone (CRH) exerts thyrotropic effects on cultured-pituitary cells (Larsen, et al. 1998). A direct stimulatory action of CRH was also demonstrated for amphibian, reptilian, and avian pituitary thyrotropes (reviewed by De Groef, et al. 2006).

The principal action of hypothalamic CRH in teleosts is the regulation of the stress response (Flik, et al. 2006; Huising, et al. 2004). In teleostean fishes, that lack a median eminence, neurons from the nucleus preopticus (NPO) send axons directly to the pituitary gland where they release CRH upon registration of a stressor. CRH stimulates the release of adrenocorticotropic hormone (ACTH) from corticotrope cells in the pituitary pars distalis (Metz, et al. 2004) by the activation of the CRH receptor-1 (CRH-R1, Huising, et al. 2004). ACTH is derived from pro-opiomelanocortin (POMC) through the action of prohormone convertase-1 (PC1, Benjannet, et al. 1991; Castro and Morrison 1997), and

stimulates the secretion of cortisol from the interrenal cells in the head kidney (Flik, et al. 2006).

The effect of CRH on pituitary corticotropes is modulated by CRH-binding protein (CRH-BP), a hypothalamic factor that binds CRH with a higher affinity than does CRH-R1 (Cortright, et al. 1995; Potter, et al. 1991). The primary action of CRH-BP is generally thought to be inhibitory on the CRH-induced ACTH release from pituitary corticotropes (Cortright, et al. 1995; Potter, et al. 1991; Westphal and Seasholtz 2006). In common carp, CRH-BP immunoreactivity is localized in perikarya in the recessus opticus, a hypothalamic region distinct from the NPO, and colocalises in CRH-immunopositive nerve fibers projecting from the NPO to pituitary corticotropes (Huising, et al. 2004). Beside CRH, urotensin-I (UI) and CRH-R2, two other members of the teleostean CRH system (Flik, et al. 2006), have also been implicated in the stress response of teleosts (Bernier and Craig 2005; Fryer, et al. 1983). UI has a potent thyrotropic effect on coho salmon pituitary cells, similar to CRH (Larsen, et al. 1998). However, the exact role of UI in the stress response and thyroid metabolism of teleosts has scarcely been addressed. Other members of the teleostean CRH-family are urocortin-2 and -3 orthologs (Boorse, et al. 2005; Hsu and Hsueh 2001; Lewis, et al. 2001), but, as yet, no data are available on their function in fish.

The dual function of CRH as a corticotropin- and a thyrotropin-releasing hormone suggests a functional relationship between the hypothalamo-pituitary-interrenal axis (HPI-axis) and the hypothalamo-pituitary-thyroid axis (HPT-axis). This is further corroborated by the finding, in fishes, that experimentally elevated plasma cortisol or thyroid hormone levels correlate negatively with plasma T4 and cortisol, respectively (Mustafa and MacKinnon 1999; Redding, et al. 1984). These findings, and in particular the absence of a thyrotropic effect of TRH in carp and the thyrotropic effect of CRH and UI in coho salmon, led us to postulate that thyroid hormones may have important regulatory effects on mediators of stress axis activity in the central nervous system of common carp.

Materials and methods

Animals and animal procedures

Common carp (*Cyprinus carpio* L.), hereafter called carp, of the all-male E4×R3R8 isogenic strain (Bongers, et al. 1997) and with a body weight of 41 ± 12 g (mean ± s.d.), were obtained from the Department of Fish Culture and Fisheries of Wageningen University (The Netherlands). Fish were kept in stock in 150 l tanks, with circulating filtered Nijmegen city tap water at 22°C, at a photoperiod ratio of 16 h light:8 h darkness. Fish were fed commercial fish food (Trouvit; Trouw, Putten, The Netherlands) at a ration of 1.5% of the estimated body weight per day.

Fish were injected intraperitoneally (i.p.) on day 1 of the treatment regimen with 10 μg T4 (from Sigma Chemical Co., St. Louis, MO, US) per gram body weight in saline (0.9% NaCl), followed by i.p. injections of 1 μg T4 per gram body weight every third day for a period of 2 weeks. Control fish were injected with vehicle only. Fish were sampled on the first day after administration of the final injection. To this end, fish were deeply anaesthetised in 0.1% 2-phenoxyethanol; mixed arterial and venous blood was collected by puncture from the caudal vessels, using heparinised 23 gauge needles. Animals were then killed by spinal transection, and pituitary gland, hypothalamus and preoptic region (hereafter referred to as hypothalamus), and head kidneys were dissected. Plasma was obtained from whole blood by centrifugation (15 min, 15000 *g*, 4°C), and stored at -20°C until further analysis. Pituitary glands used for pituitary hormone content measurements, and those used for measurements of tissue POMC, PC1, CRH-R1 and CRH-R2 mRNA levels were bisected into pars distalis and pars intermedia. Pituitary glands used for measurements of TSH β-subunit mRNA were left intact. Tissues were immediately frozen in dry ice and stored at -80°C until further processing.

In vitro superfusion and static incubation of carp head kidney

Head kidneys from two untreated carp were removed directly after anesthesia and placed individually in a superfusion chamber. Tissues were superfused with a Hepes-Tris-buffered medium (pH 7.4) saturated with carbogen (95% O_2-

5% CO_2), at a rate of 30 µl/min as described in detail by Metz, et al. (2005). When a stable baseline of cortisol secretion was obtained, T4 (at 1 µM), 3,5,3'-triiodothyronine (T3; 1 µM), or human $ACTH_{(1-39)}$ (hACTH; 0.1 µM), were administered for a period of 30 min. Ten- or twenty-minute fractions were collected, stored on ice and analysed for cortisol content as described later. Thyroid hormones and hACTH were obtained from Sigma Chemical Co.

Head kidneys from five untreated carp were obtained. The head kidneys obtained from one animal were pooled, diced into approximately 1 mm^3 sized fragments, and suspended in the same buffer that was used for superfusion experiments. The suspension was distributed in a 12-well plate in 2 ml volumes. Ten nM T4 were added; control incubations received the saline vehicle only. After 16 h, the incubation medium was sampled for cortisol, after which 30 nM hACTH were added and left to incubate for 1 h, subsequently, the medium was sampled again.

RNA extraction and cDNA synthesis

To extract total RNA, selected tissues were removed from seven animals from the control and T4-treated group respectively, and homogenised in 500 µl TRIzol reagent (Invitrogen, Carlsbad, CA, US) by ultrasonification. Following a DNase treatment (Invitrogen), 1 µg of RNA was reverse transcribed to cDNA in a 20 µl reaction mixture containing 300 ng random primers, 0.5 mM dNTPs, 10 mM dithiothreitol, 10 U RNase Inhibitor, and 200 U Superscript II Reverse Transcriptase (Invitrogen) for 50 min at 37°C and stored at -20°C.

Cloning and sequencing of carp PC1 cDNA

Primer sequences used are shown in Table 1, oligonucleotides were purchased from Biolegio (Malden, The Netherlands). PC1 sequences were retrieved from the puffer fish (*Takifugu rubripes*) and zebrafish (*Danio rerio*) genomes via a BLAST search at the Ensembl site (http://www.ensembl.org) using mouse PC1 cDNA as a query sequence. Mouse PC1 cDNA was aligned with the retrieved puffer fish and zebrafish sequences, and degenerate primers PC1-fw and PC1-rv were designed based on conserved sequences. To obtain a carp PC1

nucleotide sequence, a PCR was carried out on carp pituitary pars distalis cDNA using primers PC1-fw and PC1-rv. PCR products were separated by electrophoresis on a 1% agarose-ethidium bromide gel, ligated into a pCR4-TOPO vector, and introduced into competent TOP10 *Escherichia coli* cells (TOPO TA Cloning Kit; Invitrogen). Successfully transfected cells were selected on LB-kanamycine agar and checked for appropriately sized inserts using primers T3 and T7. Successfully transfected plasmids were extracted (Miniprep; Biorad, Hercules, CA), sequenced using primers T3 and T7, and analysed on an ABI Prism 310 automated sequencer (PE Applied Biosystems, Foster City, CA, USA). The 5′- and 3′-ends were obtained by rapid amplification of cDNA ends (RACE)-PCR using the GeneRacer kit (Invitrogen). One microgram of RNA from carp pituitary pars distalis was processed according to the GeneRacer protocol (Invitrogen) to obtain RACE-ready cDNA. PCR was performed using gene-specific primers (PC1-3′RACE, PC1-5′RACE) and the GeneRacer ligated primers. Template DNA was mixed with 600 nM GeneRacer primer and 200 nM gene-specific primers in a volume of 50 µl containing 1.75 mM $MgCl_2$, 350 µM dNTPs and 3.75 U *Taq* polymerase-*Tgo* polymerase mix (Roche Applied Science, Penzberg, Germany). The 5′- and 3′-nested PCRs (primers: PC1-3′RACEn, PC1-5′RACEn) were then performed on the PCR products to further amplify the PC1 cDNA fragments. Nested PCR products were analysed on agarose gel, cloned into a TOPO vector, and sequenced as described previously. The authenticity of the consensus sequence from four to six clones was confirmed from alignments with PC1 cDNAs from angler fish (Roth, et al. 1993) and mouse, and the putative PC1 cDNAs from the puffer fish and zebrafish genomes. The carp PC1 nucleotide sequence thus obtained has been submitted to the DDBJ-EMBL-GenBank databases under accession number AM236095.

Hormone measurements

Pituitary pars distalis and pars intermedia tissues, obtained from five to seven animals, were homogenised on ice in 200 µl 10 mM HCl in a Potter-Elvehjem device. Homogenates were diluted in demineralised water 200-fold before analysis. Blood plasma, obtained from 19 control animals and 17-21 T4-treated animals, was diluted fivefold in RIA buffer prior to cortisol measurements,

plasma analysed for α-melanocyte-stimulating hormone (MSH) was not diluted. ACTH was measured using RIA as described by Metz, et al. (2004). A commercially available polyclonal rabbit antiserum against human $ACTH_{1-24}$ (Biogenesis, Poole, UK) was used. The α-MSH RIA was performed as described by van den Burg, et al. (2003). The antiserum had full cross-reactivity with des-, mono- and di-acetylated α-MSH (Vaudry, et al. 1978). Radiolabelled ACTH and α-MSH were prepared by ^{125}I-addition using the Iodogen method (ICN, Costa Mesa, US), and purified through solid-phase extraction (octadecyl Bakerbond column, J.T. Baker, Phillipsburg, NJ, USA). Cortisol was measured by RIA, as described by Metz, et al. (2005), using a commercially available antiserum (Campro Scientific, Veenendaal, The Netherlands) and ^{125}I-cortisol (Amersham, Buckinghamshire, UK). Plasma-free T4 (fT4) was measured using a commercially available enzyme-linked immunoassay (Research Diagnostics Inc., Flanders NJ, US) according to manufacturer's instructions.

Real-time quantitative PCR

The relative tissue expression of POMC, PC1, CRH-R1, CRH-R2 and TSH β-subunit mRNA in pituitary pars distalis, pars intermedia and intact pituitary gland, obtained from seven control and seven T4-treated animals, was measured by use of real-time quantitative PCR (RQ-PCR). In hypothalamus, the relative expression of POMC, CRH, UI, prepro-TRH, PC1 and CRH-BP was measured. In general, cDNA was diluted 50-fold, but cDNA from pituitary pars distalis and pars intermedia was diluted 25000-fold to measure *POMC* gene expression. Five μl cDNA was used in a 25 μl reaction mixture consisting of 12.5 μl Sybr Green Master Mix (PE Applied Biosystems Benelux, Nieuwerkerk aan den IJssel, The Netherlands) and 3 μl of each primer (600 nM final concentration) was added. The primer sets used for PCR are shown in Table 1. RQ-PCR was performed on a GeneAmp 5700 Sequence Detection System (PE Applied Biosystems). The reaction mixture was incubated for 10 min at 95°C, followed by 40 cycles of 15 s denaturation at 95°C and 1 min annealing and extension at 60°C. Melting curves confirmed the identity and specificity of the PCR reactions. Dissociation plots were analysed and cycle threshold (C_T) values were determined. The expression of genes of interest was calculated relative to

Table 1. Primer sequences with corresponding GenBank accession numbers.

Gene prod.	Accession nr.	Primer	Sequence 5′ → 3′
PC1	AM236095	PC1-fw	AGGCTCCATYTGTKTGGG
		PC1-rv	CCASCCGGGGTTATTGGC
		PC1-3′RACE	GCACTGGCTTTGGAGCAGAATCCT
		PC1-5′RACE	GGAGATGGTGTACGGACTGTCTGT
		PC1-3′RACEn	CCTGGCGAGACCTTCAGCACTTAG
		PC1-5′RACEn	CAGTTGTCGCCCTGACGACCACCG
		qPC1-fw	GCTGGTTTGATGGTCAACAG
		qPC1-rv	GACTTTGGGATCAGCCAGAT
vector		T3	ATTAACCCTCACTAAAGGGA
		T7	TAATACGACTCACTATAGGG
TSH-β	AB003585	qTSHβ-fw	AGTACCGAACGGCCATCTTG
		qTSHβ-rv	TGCCACCGGATAGGTGAAGT
POMC	Y14618	qPOMC-fw	TTGGCTCTGGCTGTTCTGTGT
		qPOMC-rv	TCATCTGTCAGATCAGACCTGCATA
CRH-R1	AJ576244	qCRH-R1-fw	CCCTGCTGATCGCCTTCAT
		qCRH-R1-rv	GCAGGATAAATGCTGTAATCAGGTT
CRH-R2	AJ781795	qCRH-R2-fw	GTTGGAGCTCTTGTTATTGCCTTTA
		qCRH-R2-rv	GAAAGTCGTAATTAAGTTCCAGTGGAT
CRH	AJ317955	qCRH-fw	CATCCGGCTCGGTAACAGAA
		qCRH-rv	CCAACAGACGCTGCGTTAACT
UI	M11671	qUI-fw	GCACCTGTGTCCAGCATGAA
		qUI-rv	GGTGCTCAGCGGGATGTG
pp-TRH	AB179818	qTRH-fw	TTACCGGTCGCTTTCACGTT
		qTRH-rv	AGCTGTGTGCCAAACCAAAC
CRH-BP	AJ490881	qCRH-BP-fw	ACAATGATCTCAAGCGGTCCAT
		qCRH-BP-rv	CCACCCAGAAGCTCGACAAA
β-actin	M24113	qACT-fw	CAACAGGGAAAAGATGACACAGATC
		qACT-rv	GGGACAGCACAGCCTGGAT
40S	AB012087	q40S-fw	CCGTGGGTGACATCGTTACA
		q40S-rv	TCAGGACATTGAACCTCACTGTCT

Y: C or T/U; S: C or G; K: G or T/U.

β-actin and 40S ribosomal protein S11 expression. Both control genes yielded similar results and therefore all results in this study are expressed relative to β-actin mRNA expression.

Statistical analysis

All data are presented as mean values ± s.e.m. The number of different preparations (n) is given in parentheses. Differences between groups were assessed with Student's unpaired t-test. Statistical significance was accepted at $P < 0.05$ (two-tailed), probabilities are indicated by asterisks (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

Results

Animal model validation

Plasma-free T4 concentrations were elevated 5.3-fold ($P < 0.001$) in T4-treated fish (Fig. 1A), and pituitary TSH β-subunit mRNA levels were 3.9-fold ($P < 0.001$) lower than in control animals (Fig. 1B), which confirmed a successful induction of hyperthyroidism. The validation of our experimental hyperthyroid animal model is further corroborated by the hyperactive behaviour of the T4-injected group, in particular during feeding, compared to the control group (personal observations).

Plasma hormone levels

In control animals, basal plasma cortisol levels were 54 ± 6 ng/ml (equivalent to 149 ± 18 nM; $n = 19$), which is similar to the values measured in unstressed carp reported elsewhere (Metz, et al. 2004; van den Burg, et al. 2003). In hyperthyroid carp, basal plasma cortisol levels were reduced 3.2-fold ($P < 0.001$; Fig. 2A). Plasma α-MSH levels in control carp were 0.30 ± 0.11 nM ($n = 19$), which values are, again, within the range measured in unstressed carp (Metz, et al. 2005; van den Burg, et al. 2003; van den Burg, et al. 2005a). In hyperthyroid carp, basal α-MSH levels had increased by 30% ($P < 0.05$; Fig. 2B).

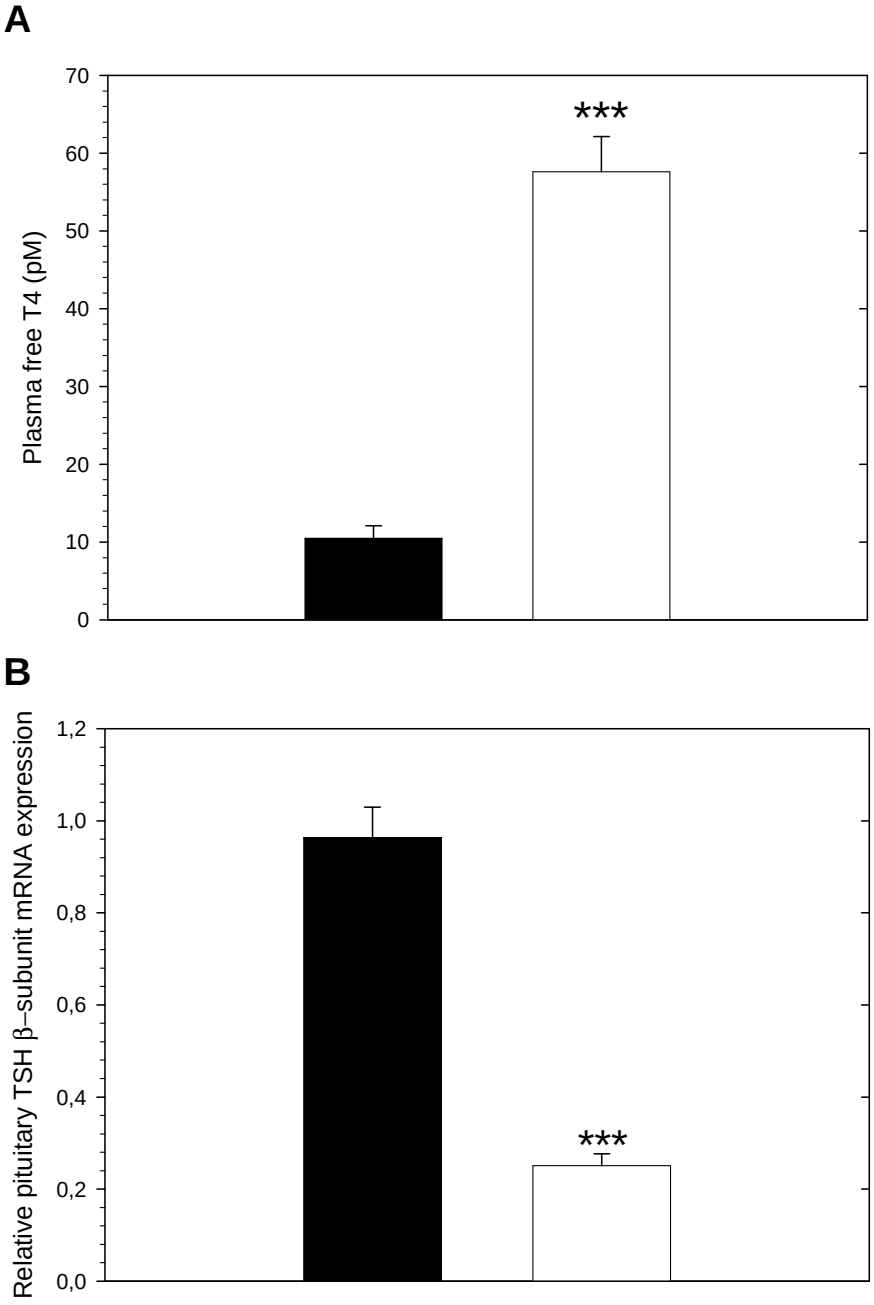

Figure 1. A. Plasma free T4 concentrations (pM) in saline-injected (closed bar, *n*=19) and T4-injected (open bar, *n*=21) animals. **B**. Expression levels of TSH β-subunit mRNA relative to β-actin in the pituitary glands of saline-injected (closed bar, *n* = 7) and T4-injected (open bar, *n* = 7) animals. Student's *t*-test was used for statistical evaluation.

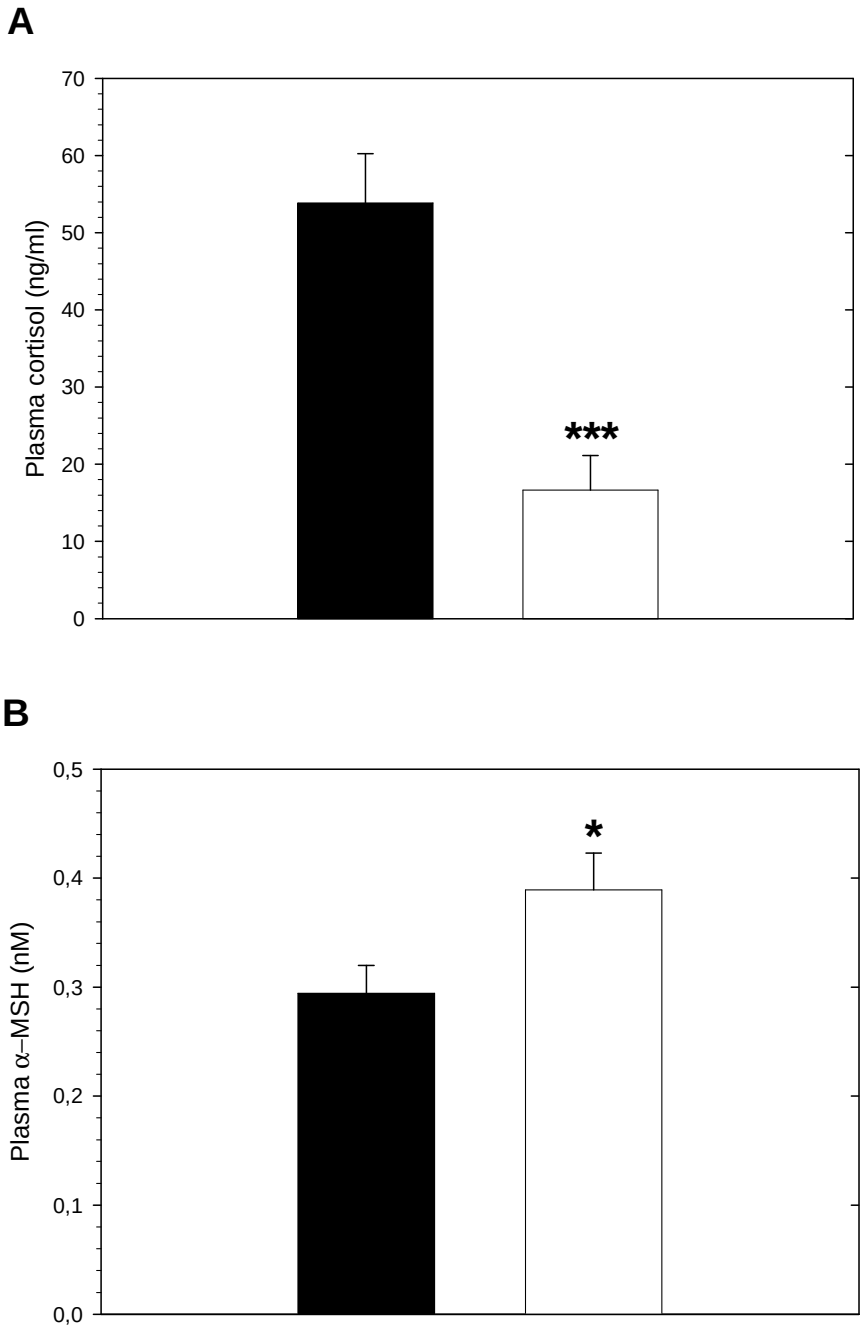

Figure 2. A. Plasma cortisol and **B.** α-MSH concentrations in saline-injected (closed bars, n = 19) and T4-injected (open bars, n = 21 and 17 respectively) animals. Student's t-test was used for statistical evaluation.

Pituitary pars distalis and hypothalamus

In the pituitary pars distalis, no significant changes in POMC, PC1 and CRH-R1 mRNA expression levels relative to β-actin were detected between saline-injected and T4-injected animals, whereas CRH-R2 mRNA was not detectable (n = 7, results not shown). Concomitantly, the ACTH content of the pituitary pars distalis was similar in saline-injected and T4 injected carp (189 ± 16 and 176 ± 23 pg/gland, respectively; n = 7; P = 0.65). In the hypothalamus, mRNA levels of POMC, CRH, UI, prepro-TRH and PC1 were not affected by the T4-treatment, but CRH-BP mRNA levels were elevated significantly by 40% in hyperthyroid animals ($P < 0.05$; Fig. 3).

Pituitary pars intermedia

In the pituitary pars intermedia of hyperthyroid carp POMC and PC1 mRNA expression levels were elevated 1.5-fold ($P < 0.05$) and 2.4-fold ($P < 0.01$) respectively; whereas the expression of CRH-R1 mRNA was not affected by T4 treatment. CRH-R2 mRNA was not detectable (Fig. 4). The α-MSH content of the pars intermedia of hyperthyroid animals was 30 ± 5 pmol/gland (n = 5), and had not changed significantly compared with controls, whose value was 32 ± 4 pmol/gland (n = 7; P = 0.77).

In vitro head kidney incubations

In two pilot experiments, carp head kidneys superfused *in vitro* displayed a basal cortisol secretion rate of 58 ± 9 pg/min per head kidney, which is similar to values reported earlier (Metz, et al. 2005). Following a 20-min exposure to 1 μM T4 or T3, the basal cortisol release was virtually unchanged; the viability of the head kidney tissue was confirmed by stimulation with 0.1 μM hACTH, to which the tissue responded with a pronounced rise in the cortisol secretion rate (results not shown). Based on these observations we decided to perform long-term incubations of head kidneys with thyroid hormones. After a 16-h static incubation, the cortisol concentration in the control incubation medium was 0.26 ± 0.03 nM (n = 5). When head kidney tissue was incubated in the presence

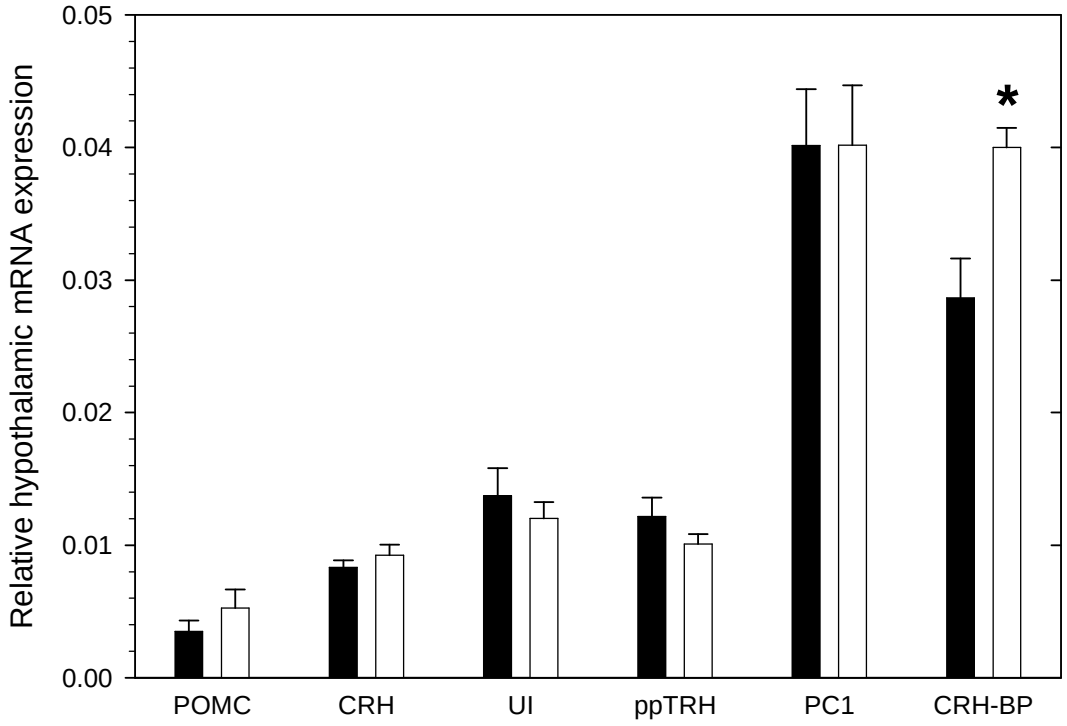

Figure 3. Expression levels of POMC, CRH, urotensin-I (UI), prepro-TRH (ppTRH), PC1 and CRH-BP mRNA relative to β-actin in hypothalamic tissue of saline-injected (closed bars, $n = 7$) and T4-injected (open bars, $n = 7$) animals. Student's t-test was used for statistical evaluation.

of 10 nM T4 for the same period of time, the cortisol concentration in the incubate was similar: 0.27 ± 0.07 nM (n = 5; P = 0.86). Upon subsequent incubation with 0.1 μM hACTH for 1 h, the cortisol concentration in the control medium had increased to 4.4 ± 1.3 nM (n = 5), and that in the medium containing 10 nM T4 to 3.1 ± 1.8 nM (n = 5; P = 0.59), confirming the viability of the head kidney tissue after a prolonged static incubation.

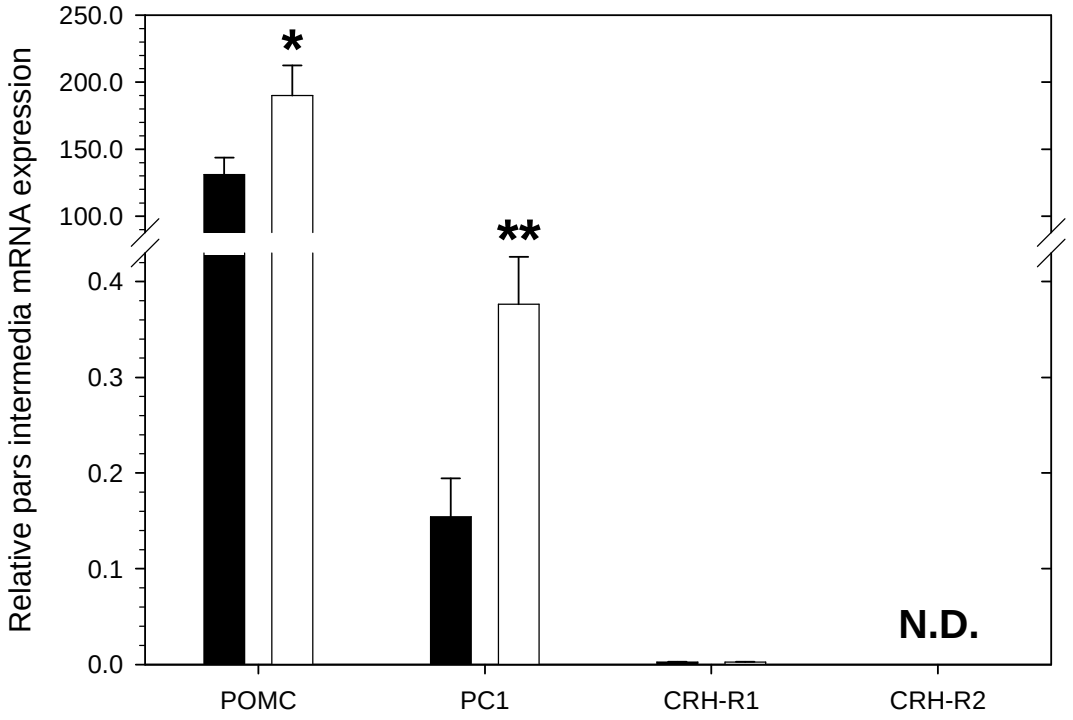

Figure 4. Expression levels of POMC, prohormone convertase-1 (PC1), CRH-R1 and CRH-R2 mRNA relative to β-actin in the pituitary pars intermedia in saline injected (closed bars, n = 7) and T4 injected (open bars, n = 7) animals. N.D.: not detectable. Student's t-test was used for statistical evaluation.

Discussion

Basal plasma cortisol levels in the control group were similar to the values measured in unstressed carp in other studies (Metz, et al. 2004; van den Burg, et al. 2003). Moreover, the basal cortisol levels we measured, one day after the final i.p. injection was administered, are considerably lower than the concentrations that were measured in carp subjected to an acute or chronic stressor, which displayed plasma cortisol levels that were increased 5- to 26-fold (Huising, et al. 2004; Metz, et al. 2004; van den Burg, et al. 2003; Weyts, et al. 1997) and 5- to 10-fold (Huising, et al. 2004; Metz, et al. 2005), respectively, compared to control levels. It was also observed that after the removal of an acute stressor, the elevated plasma cortisol levels in carp decrease rapidly, *i.e.* within 90-120 min, to basal control levels (Huising, et al. 2004; Weyts, et al.

1997), and that no changes occur in the hypothalamic expression of CRH and CRH-BP (Huising, et al. 2004). We judge that our injection protocol *per se* did not constitute a chronic stressor, and did not result in acclimation of the experimental animals. The cortisol concentrations that were measured in the control group reflect basal, unstressed levels.

We here demonstrate in a validated experimental animal model that experimentally induced hyperthyroidism results in a marked hypocortisolemia. The decrease in basal plasma cortisol correlates with an increase in hypothalamic CRH-BP mRNA expression. Since the release of cortisol from viable carp head kidney tissue does not respond to short- and long-term exposure to T4 *in vitro*, we exclude the interrenal cells as a target for direct thyroid hormone action. Taken together, these results support a role for thyroid hormone as a regulator of the teleostean stress axis, by the modulation of CRH-BP expression and, hence, the bioavailability of ligands from the CRH family. In carp, CRH-BP is expressed in a cell population that is clearly distinct from the CRH-immunoreactive nucleus preopticus (Huising, et al. 2004), and these neurons could well be the target for thyroid hormone action.

Our experimental design only allowed the determination of end-point measurements. Earlier changes, if present, in the CRH and thyroid systems of our animals would therefore not be detected. Indeed, van den Burg, et al. (2005b) exposed carp to an acute change in ambient temperature and demonstrated, using functional magnetic resonance imaging, that there is a fast response (*i.e.* already evident after 30 s) and a clear chronology in the activation and deactivation of brain areas that are involved in the stress response. On a time scale of hours to days, chronically stressed carp differentially express members of the CRH system, and have blunted, but still elevated plasma cortisol levels (Huising, et al. 2004; Metz, et al. 2005) which reflect acclimation of the fish to the stressor. Our results can then be interpreted as the acclimation of the CRH and thyroid systems to a chronic hyperthyroidism. We are presently designing time-course studies to investigate the dynamics of the interactions between the CRH and thyroid systems.

The activation of the hypothalamo-pituitary-interrenal (HPI) axis in teleosts results in the release of cortisol, the prime stress hormone. The main function of cortisol in the stress response of teleostean fish is the redistribution

of energy away from, *e.g.*, growth and reproduction, and toward physiological functions required for coping with the stressor and to restore homeostasis (Schreck 2000; Wendelaar Bonga 1997). Cortisol stimulates gluconeogenesis and lipolysis (Sheridan 1988; van der Boon, et al. 1991), which result in increased plasma glucose and free fatty acid levels. These catabolic actions of cortisol reflect its glucocorticoid potency that is commensurate to the central role of this hormone in the stress response and the regulation of energy metabolism.

As is cortisol, thyroid hormone is fundamentally implicated in the regulation of energy metabolism in vertebrates. Thyroid hormone stimulates basal metabolic rate and oxygen consumption in several teleostean tissues (Lynshiang and Gupta 2000; Peter and Oommen 1989), and has profound effects on lipid, carbohydrate and protein metabolism in teleosts (Ballantyne, et al. 1992; Plisetskaya, et al. 1983; Scott-Thomas, et al. 1992; Shameena, et al. 2000; Sheridan 1986). In addition, during prolonged fasting, plasma T3 levels decrease in Nile tilapia (*Oreochromis niloticus*, Van der Geyten, et al. 1998) which could well represent a mechanism for the adaptation, by downregulating energy expenditure, to caloric and nutritional deprivation.

Since both the HPI- and HPT-axis are fundamentally involved in the regulation of energy expenditure, it can be anticipated that, of necessity, mutual interactions between these two exist. Indeed, European eel (*Anguilla anguilla*) and coho salmon (*Oncorhynchus kisutch*) treated with cortisol showed suppressed plasma T3 levels (Redding, et al. 1986; Redding, et al. 1984). It is interesting to note that Larsen, et al. (1998) demonstrated thyrotropic effects of CRH-family peptides in cultured pituitary cells from the latter species. Two other salmonid species, Atlantic salmon (*Salmo salar*) and arctic charr (*Salvelinus alpinus*), treated with cortisol or given iodide supplementation, showed predictably increased plasma cortisol and thyroid hormone levels respectively, and that correlated negatively with plasma thyroid hormone and cortisol levels respectively (Mustafa and MacKinnon 1999). We here suggest that these negative correlations between plasma cortisol and thyroid hormone are caused by the interaction of the HPI- and HPT-axis in the brain of the fish. This would allow the integration of afferent signals and the coordinated regulation of, ultimately, the interrenal cells and thyroid gland.

To our knowledge, this is the first study to simultaneously investigate the *in vivo* responsiveness of both the CRH system and TRH for treatment with thyroid hormone in a teleost. The upregulation of hypothalamic *CRH-BP* gene expression in hyperthyroid carp suggests that a member of the CRH family is the principal TSH-releasing factor in this species. This is further corroborated by the non-responsiveness of prepro-TRH expression and PC1 that is involved in the processing of pro-TRH to mature TRH in the hypothalamus (Schaner, et al. 1997). Indeed, in carp (Kagabu, et al. 1998) and a number of other teleosts (Gorbman and Hyder 1973; Melamed, et al. 1995), TRH does not stimulate TSH secretion (Larsen, et al. 1998). Instead, CRH is the thyrotropic factor. CRH has a widespread distribution in the teleostean brain; hypophysiotropic CRH-positive neurons have been identified in the NPO, and the nucleus lateralis tuberis (NLT) and nucleus recessi lateralis (NRL) in the hypothalamus (Huising, et al. 2004; Matz and Hofeldt 1999; Pepels, et al. 2002). Matz & Hofeldt (1999) showed that, in Chinook salmon (*Oncorhynchus tshawytscha*), CRH-immunoreactive fibers, originating from the preoptic region and NLT, terminate with bouton-like swellings in the proximity of TSH-ir cells in the pituitary pars distalis. These anatomical data, together with our results provide evidence for the existence of a CRH-TSH-T4 axis in teleosts. However, since UI is very potent in stimulating the secretion of TSH from cultured teleostean pituitary cells (Larsen, et al. 1998), we cannot exclude UI as a thyrotropic principle. Yulis, et al. (1986) showed extensive UI-ir staining in hypothalamic perikarya as well as in fibers that innervate the pituitary proximal pars distalis of the white sucker (*Catostomus commersoni*). Indeed, in carp (our unpublished results) and other teleosts (Ueda, et al. 1983), the proximal pars distalis harbours TSH-ir cells. Since CRH and UI have a high affinity for CRH-BP (Baigent and Lowry 2000; Valverde, et al. 2001), an increased expression of CRH-BP is likely to affect the bioavailability of UI as well as that of CRH. Experimental data clearly point to UI as a thyrotropic factor, and, until matters are conclusively resolved, the teleostean HPT-axis should be represented as a CRH/UI-TSH-T4 axis.

TRH also has a wide distribution in the teleostean brain, and hypophysiotropic neurons expressing TRH were found in distinct parvocellular and magnocellular regions in the NPO of salmonids (Ando, et al. 1998; Díaz, et al. 2001). In carp brain, TRH-ir neurons are located in the NRL, and TRH-ir

fibers are present in the NLT and NPO (Hamano, et al. 1990), which nuclei, in the same animal species, also express CRH (Huising, et al. 2004). By their anatomical locations, it thus appears that hypothalamic CRH-ir and TRH-ir neurons can communicate bi-directionally.

We found that experimental hyperthyroidism resulted in an increase in plasma α-MSH, which was corroborated by increased pituitary pars intermedia POMC and PC1 mRNA expression. Although α-MSH is considered to be corticotropic in Mozambique tilapia *(Oreochromis mossambicus*, Balm, et al. 1995; Lamers, et al. 1992) no stimulatory effect of α-MSH was observed on carp interrenal cells *in vitro* (Metz, et al. 2005). It was suggested that elevated plasma α-MSH levels sustain HPI-axis activity through a short-loop positive feedback on CRH neurons in the nucleus preopticus (Metz, et al. 2005). The opposite changes in plasma cortisol and α-MSH levels in hyperthyroid carp reported here are compatible with a role of α-MSH in the fine-tuning of HPI-axis activity, but our results do not provide conclusive evidence.

We here postulate that the effects observed in this study are likely related via the physiological actions of cortisol and thyroid hormone in the regulation of energy metabolism. α-MSH can fulfil a similar role, although evidence is scarce. In goldfish (*Carassius auratus*), POMC mRNA expression was observed in brain regions known to control food intake, and intracerebroventricular injection of the α-MSH agonist [Nle^4, D–Phe^7]-α-MSH (NDP-α-MSH) inhibited food intake (Cerdá-Reverter, et al. 2003). We interpret our experimental results to reflect the concerted actions of cortisol, thyroid hormone and α-MSH on energy metabolism in carp.

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Chapter 5

Central and peripheral integration of interrenal and thyroid axes signals in common carp (*Cyprinus carpio* L.)

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Abstract

In teleostean fishes the hypothalamic-pituitary-thyroid axis (HPT axis) and the hypothalamic-pituitary-interrenal axis (HPI axis) regulate the release of thyroid hormones and cortisol respectively. Since many actions of both hormones are involved in the regulation of metabolic processes, communication between both signal pathways can be anticipated. In this study we describe central and peripheral sites for direct interaction between mediators of both neuroendocrine axes in the common carp (*Cyprinus carpio*). Despite suggestions in the literature that corticotropin-releasing hormone (CRH) is thyrotropic in some fish, we were not able to establish stimulatory effects of CRH on the expression of the pituitary TSH-β subunit gene. In preoptic area tissue incubated with 10^{-7} M thyroxine (T4) a 2.9-fold increase in the expression of CRH-binding protein (CRH-BP) was observed. Thus, T4 could reduce the bioavailable hypothalamic CRH via the up regulation of CRH-BP expression and hence down regulate the HPI axis. At the peripheral level, cortisol (10^{-6} M), ACTH (10^{-7} M), and α-MSH (10^{-7} M) stimulate the release of T4 from kidney and head kidney fragments, which contain all functional thyroid follicles in carp, by two- to fourfold. The substantiation of three pituitary thyrotropic factors, *viz*. TSH, ACTH and α-MSH, in common carp, allows for an integration of central thyrotropic signals. Clearly, two sites for interaction between the HPT axis, the HPI axis and α-MSH are present in common carp. These interactions may be key to proper regulation of general metabolism in this fish.

Introduction

Thyroid hormones and corticosteroids are major endocrine signals that are involved in the regulation of fundamental and basic physiological processes in vertebrates. The action of thyroid hormones is pleiotropic and often permissive, but generally contributes to the regulation of growth, metabolism, development, and metamorphosis (Blanton and Specker 2007). Glucocorticosteroids are considered to be prime stress hormones that govern the stress response through the redistribution of energy toward processes required for coping with the stressor (Wendelaar Bonga 1997). Because of the significance of thyroid hormones and corticosteroids in the regulation of metabolic processes, we postulate that a bidirectional communication between these endocrine systems is a necessity for the integration and proper functioning of either system.

In teleostean fishes, the thyroid hormone thyroxine (T4, 3,5,3'5'-tetra-iodothyronine) and T3 (3,5,3'-triiodothyronine) are the end products of the hypothalamic-pituitary-thyroid (HPT) axis where, generally, hypothalamic TRH stimulates the release of pituitary TSH, which stimulates the release of thyroid hormones (Bernier, et al. 2009). Similarly, the hypothalamic-pituitary-interrenal (HPI) axis controls the release of cortisol from the interrenal cells in the head kidney, via hypothalamic CRH and pituitary ACTH. The hypothalamic and pituitary components of both axes are inhibited through negative feedback by their respective end products. The multilevel control of thyroid hormone and cortisol release allows for potential multiple sites of interaction between both endocrine systems.

Interactions between the HPT and HPI axes have been described in teleostean fishes. Long-term and short-term exposure to cortisol or dexamethasone resulted in decreased levels of plasma thyroid hormones in several fish species (Brown, et al. 1991; Redding, et al. 1986; Redding, et al. 1984; Walpita, et al. 2007). These decreased hormone levels were associated with either increased clearance of plasma thyroid hormones (Redding, et al. 1986) or changes in the activity and expression of deiodinases (Walpita, et al. 2007). Stimulatory effects of cortisol on the HPT axis have also been suggested in teleost fish. In brook charr (*Salvelinus fontinalis*) long-term exposure to cortisol increased the hepatic conversion of T4 to T3 (Vijayan, et al. 1988) and in

Japanese flounder (*Paralichthys olivaceus*) cortisol augmented the effects of thyroid hormones on the resorption of the dorsal fin ray (de Jesus, et al. 1990). Experimental data on the effects of thyroid hormones on the HPI axis in teleosts are scarce. In pre- and post-smolt coho salmon (*Oncorhynchus kisutch*), thyroxine treatment resulted in increased and decreased sensitivity of the head kidney to ACTH, respectively (Young and Lin 1988).

In several species from all non-mammalian vertebrate classes, CRH does not only stimulate the release of ACTH, but also that of TSH (De Groef, et al. 2006). Indeed, in common carp (*Cyprinus carpio*), CRH has been suggested to exhibit thyrotropic activity. In this species, TRH does not stimulate the release of TSH from cultured pituitary cells (Kagabu, et al. 1998). Moreover, experimental treatment with thyroxine resulted in a marked hypocortisolemia in carp, which was accompanied by an increased mRNA expression of CRH-binding protein (CRH-BP) in the preoptic area and unchanged levels of CRH and prepro-TRH mRNA (Geven, et al. 2006). It appears that, in common carp, not TRH but CRH is controlling the activity of the thyroid gland. Because of its corticotropic and putative thyrotropic activity, CRH neurons may constitute a central site for the communication between the HPT as the HPI axis in common carp.

An investigation on the location of the thyroid gland in common carp revealed another putative site for the integration of HPT and the HPI axes signals. Whereas in most fishes the thyroid follicles are located in the subpharyngeal region, surrounding the ventral aorta, in common carp all functional thyroid follicles, as characterised by iodine uptake and TSH-mediated T4 release, are scattered throughout the kidney and head kidney (Geven, et al. 2007). The close juxtaposition in the head kidney of thyroid hormone-producing follicles to cortisol-producing interrenal cells strongly hints at a paracrine interaction between both endocrine tissues.

Besides the apparent communication between HPT and HPI axes signals in common carp, a third endocrine signal appears to be involved in these axes. Plasma levels of α-MSH are increased in hyperthyroid and stressed carp (Geven, et al. 2006; Metz, et al. 2005), the exact physiological role of which still is unclear. We hypothesise that in common carp the preoptic area and the head kidney represent a central and a peripheral site respectively, for the integration

of signals of the HPT and the HPI axis. In this study, we have investigated this hypothesis by performing *in vitro* incubations of preoptic area tissues, pituitary glands, and renal tissues with several mediators of both neuroendocrine systems, including α-MSH.

Materials and methods

Animals

Common carp (*Cyprinus carpio*), hereafter called carp, of the all-male E4×R3R8 isogenic strain (Bongers, et al. 1998) were obtained from the Department of Fish Culture and Fisheries of Wageningen University (The Netherlands). Fish were kept in 140 l tanks with aerated, circulating, city of Nijmegen tap water, at a photoperiod of 16 h light:8 h darkness at 23°C. Carp were fed Trouvit dry food pellets (Trouw Nutrition International, Putten, The Netherlands) once daily at a ration of 1.5% of the estimated body weight. Before collection of tissues, fish were deeply anesthetised with 0.1% (v/v) 2-phenoxyethanol and killed by spinal transection. Animal handling followed approved university guidelines.

Static incubation of preoptic area and pituitary gland

The pituitary gland and the preoptic area containing the nucleus preopticus (NPO) were dissected as described by Metz and colleagues (Metz, et al. 2006a). The preoptic areas were diced in ~ 2 mm^3 sized fragments, while the pituitary glands were kept intact. The quality of dissection was assured by stereomicroscopic analysis. The preoptic area fragments and pituitary glands were immediately transferred to 1 ml ice-cold Leibovitz's L-15 medium (Invitrogen, Carlsbad, CA, USA) containing 100 μg/ml kanamycin (Invitrogen) and antibiotic/antimycotic (1 ×) (Invitrogen). After 1 h, the preoptic area fragments were carefully transferred to 1 ml fresh L-15 medium supplemented with T4 (at concentrations of 10^{-8} and 10^{-7} M respectively), while the pituitary glands were transferred to 1 ml fresh L-15 medium supplemented with T4 (10^{-8} and 10^{-7} M), ovine (o)CRH (10^{-7} M), or human (h)TRH (10^{-7} M), L-15 medium of

controls did not receive any supplement. Thyroxine, oCRH and hTRH were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Preoptic area fragments and the pituitary glands were incubated for periods as indicated in the legends to the figures. All tissues were incubated at 22°C, while continuously shaking (200 r.p.m.). Each medium was replaced at 1, 6, 12 and 24 h after the start of incubation. After the incubation, the preoptic area fragments and pituitary glands were immediately stored at -80°C until further processing.

RNA extraction and cDNA synthesis

To extract total RNA, the preoptic area fragments and pituitary glands were homogenised in 500 µl TRIzol reagent (Invitrogen) by ultrasonification. Following treatment with DNase, 1 µg of RNA was reverse transcribed to cDNA in a 20 µl reaction mixture containing 300 ng random primers, 0.5 mM dNTPs, 10 mM dithiothreitol, 10 U RNase Inhibitor and 200 U Superscript II Reverse Transcriptase (Invitrogen) for 50 min at 37°C and stored at -20°C.

Real-time quantitative PCR

Since homologous antibodies against the peptides we wished to quantify are not available for carp, and heterologous antibodies are validated for qualitative purposes only, we measured the expression of CRH, prepro-TRH (ppTRH), urotensin I (UI) and CRH-BP mRNA in the preoptic area and of TSH β-subunit, proopiomelanocortin (POMC), prohormone convertase 1 (PC1) and prolactin (PRL) mRNA in pituitary gland by real-time quantitative PCR (RQ-PCR). In general, cDNA was diluted ten times, but a portion of pituitary gland cDNA was diluted 500 times to measure POMC gene expression. Totally 5 µl cDNA was used in a 25 µl reaction mixture consisting of 12.5 µl Sybr Green Master Mix (PE Applied Biosystems Benelux, Nieuwerkerk aan den IJssel, The Netherlands) and 3 µl of each primer (600 nM final concentration). The primer sets used for PCR are shown in Table 1. The RQ-PCR was performed on a GeneAmp 5700 Sequence Detection System (PE Applied Biosystems). The reaction mixture was incubated for 10 min at 95°C, followed by 40 cycles of 15 s

Table 1. Primer sequences with corresponding GenBank accession numbers. Open reading frame positions are relative to the start codon.

Gene product	Accession number	Primer	Open reading frame position	Sequence 5′ → 3′
CRH	AJ317955	qCRH-fw	162 – 181	CATCCGGCTCGGTAACAGAA
		qCRH-rv	277 – 257	CCAACAGACGCTGCGTTAACT
ppTRH	AB179818	qTRH-fw	950 – 969	TTACCGGTCGCTTTCACGTT
		qTRH-rv	1079 – 1060	AGCTGTGTGCCAAACCAAAC
UI	M11671	qUI-fw	-15 – 5	GCACCTGTGTCCAGCATGAA
		qUI-rv	69 – 52	GGTGCTCAGCGGGATGTG
CRH-BP	AJ490880	qCRH-BP-fw	680 – 701	ACAATGATCTCAAGCGGTCCAT
		qCRH-BP-rv	746 – 727	CCACCCAGAAGCTCGACAAA
TSH-β	AB003585	qTSHβ-fw	230 – 249	AGTACCGAACGGCCATCTTG
		qTSHβ-rv	297 – 278	TGCCACCGGATAGGTGAAGT
POMC	Y14618	qPOMC-fw	40 – 60	TTGGCTCTGGCTGTTCTGTGT
		qPOMC-rv	176 – 152	TCATCTGTCAGATCAGACCTGCATA
PC1	AM236095	qPC1-fw	1300 – 1319	GCTGGTTTGATGGTCAACAG
		qPC1-rv	1381 – 1361	GACTTTGGGATCAGCCAGAT
β-actin	M24113	qACT-fw	342 – 366	CAACAGGGAAAAGATGACACAGATC
		qACT-rv	424 – 406	GGGACAGCACAGCCTGGAT
40S	AB012087	q40S-fw	365 – 384	CCGTGGGTGACATCGTTACA
		q40S-rv	433 – 410	TCAGGACATTGAACCTCACTGTCT

denaturation at 95°C and 1 min annealing and extension at 60°C. Analysis of dissociation plots confirmed the specificity of the PCRs. Cycle threshold values were determined from amplification curves. The expression of genes of interest was calculated relative to 40S ribosomal protein S11 mRNA expression.

Static incubation of kidney and head kidney

Head kidneys and kidney tissue were removed from the animal, diced into ~ 2 mm^3 sized fragments and immediately placed in appropriate volume of ice-cold Leibovitz's L-15 medium (Invitrogen) containing 100 µg/ml kanamycin (Invitrogen) and antibiotic/antimycotic (1 ×) (Invitrogen). After 1 h, the head kidney and kidney fragments were carefully transferred to 2 ml of fresh L-15

medium supplemented with cortisol (at concentrations of 10^{-7} and 10^{-6} M respectively) human ACTH (10^{-7} M) or monoacetyl α-MSH (10^{-7} M), while control incubations did not receive any supplement. Cortisol, hACTH and α-MSH were from Sigma Chemical Co.

Tissues were incubated for 24 h at 22°C, while continuously shaking (200 r.p.m.), after which the 2 ml incubation medium was separated from the tissue by centrifugation (4°C, 1000 *g*, 15 min) and reduced to 0.5 ml by vacuum drying. The incubation medium was applied to a Sephadex LH-20 column to isolate the thyroid hormones and to remove salts (Mol and Visser 1985). In short, glass pipettes were filled with 1 ml Sephadex LH-20 (Amersham Biosciences, Uppsala, Sweden) suspension in water (10% w/v) and equilibrated with 3 volumes of 1 ml 0.1 M HCl. Samples were acidified with an equal volume of 1 M HCl and loaded on to the column. The samples were then eluted from the column with 5 volumes of 1 ml 0.1 M HCl for the removal of ions, 4 volumes of 1 ml H_2O to neutralise the column, and 3 volumes of 1 ml 0.1 M NH_3/EtOH to collect thyroid hormones. The fractions containing thyroid hormones were vacuum dried and reconstituted in 60 µl 50 mM sodium barbitone/0.1% BSA buffer (pH 8.6). Total thyroxine was measured in duplicate with a total T4 ELISA (Human Gessellschaft für Biochemica und Diagnostica GmbH, Wiesbaden, Germany) according to the manufacturer's instruction. Standards were prepared in the same barbitone buffer as the samples were. The intra-assay and inter-assay coefficients of variation for the tT4 ELISA reported by the manufacturer are 4.2 and 3.3%, respectively. The reported cross reactivity of the ovine anti-T4 antibody to D-T4 is 98% (the reactivity to L-T4 is set at 100% as a reference), and to L-T3 and D-T3 is 3 and 1.5% respectively. Cross-reactivities of the antibody to diiodothyronine, diiodotyrosine and iodotyrosine are < 0.01%. The reported sensitivity of the tT4 ELISA is 4 ng/ml T4. Protein content of the incubated tissues was determined by Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA) using BSA as reference.

Statistical analysis

All data are represented as mean values ± s.d. The number of different preparations (*n*) is given in parentheses. Differences between groups were

assessed with Student's parametric *t*-test for unpaired observations, or Mann-Whitney's non-parametric *U*-test, where appropriate. Statistical significance was accepted at $P < 0.05$ (two-tailed), probabilities are indicated by asterisks (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

Results

Effects of CRH and TRH on pituitary TSH-β gene expression

Thyroxine, at concentrations of 10^{-8} and 10^{-7} M, significantly down regulated the expression of TSH β-subunit mRNA by 35% ($P = 0.01$) and 45% ($P = 0.02$) compared to control incubations, respectively (Fig. 1A), which demonstrated the viability of the pituitary gland preparation. Neither TRH (10^{-7} M) nor CRH (10^{-7} M) altered TSH β-subunit gene expression (Fig. 1B, C). Incubation of pituitary glands with TRH (10^{-7} M) increased the expression of POMC ($P = 0.04$) and PC1 ($P = 0.002$) 1.7- and 2.3-fold, respectively (Fig. 2D), which confirmed the bioactivity of TRH. The integrity and bioactivity of the CRH preparation used was confirmed in our laboratory by mass spectrometry and the stimulatory action on the release of ACTH and α-MSH from carp pituitary glands *in vitro* (Metz, et al. 2004; van den Burg, et al. 2005).

Effects of T4 on gene expression in the preoptic area

Thyroxine, at 10^{-7} M, significantly increased the expression of CRH, prepro-TRH and CRH-BP in the preoptic area 4.6- ($P = 0.0001$), 2.9- ($P = 0.002$) and 2.1-fold ($P = 0.04$), respectively (Fig. 2). The expression of UI remained unchanged (Fig. 2D). Incubation with 10^{-8} M T4 had no statistically significant effects on the expression of any of the genes tested (Fig. 2).

Effects of cortisol, ACTH and α-MSH on the release of T4 from renal tissues

Cortisol at 10^{-6} M, but not at 10^{-7} M, stimulated the release of T4 from head kidney ($P = 0.04$) and kidney tissue ($P = 0.01$) 3.5-fold (Fig. 3). ACTH and α-MSH (both at 10^{-7} M) increased the release of T4 from head kidney and kidney

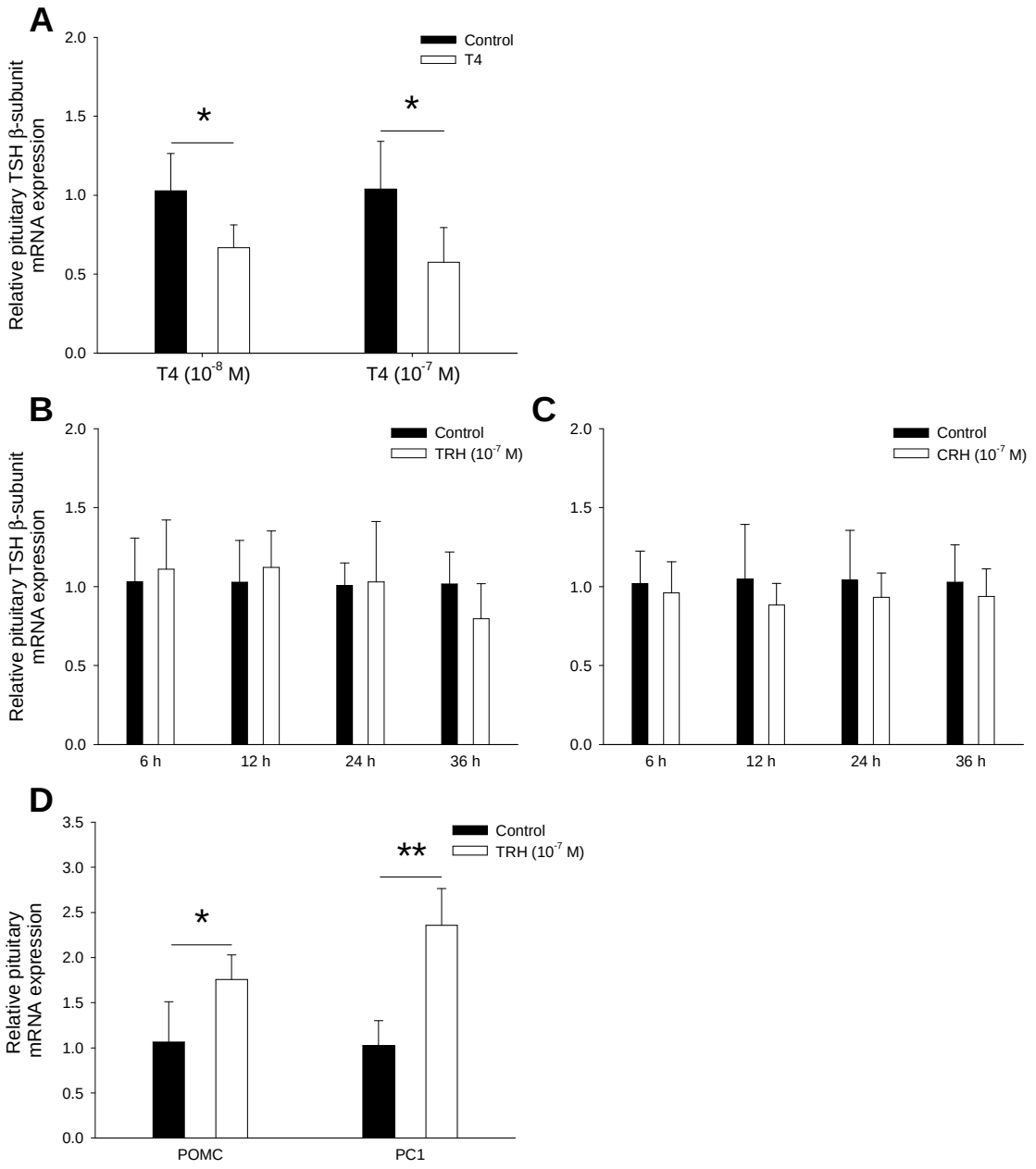

Fig 1. Relative mRNA expression levels of pituitary TSH β-subunit upon (**A**) 36 h incubation with T4 (10^{-8} M, n = 6 and 10^{-7} M, n = 5) and (**B**) 6, 12, 24 and 36h incubation with TRH (10^{-7} M, n = 4) and (**C**) CRH (10^{-7} M, n = 6). Relative mRNA expression of pituitary POMC and PC1 upon (**D**) 6 h incubation with TRH (10^{-7} M, n = 4).

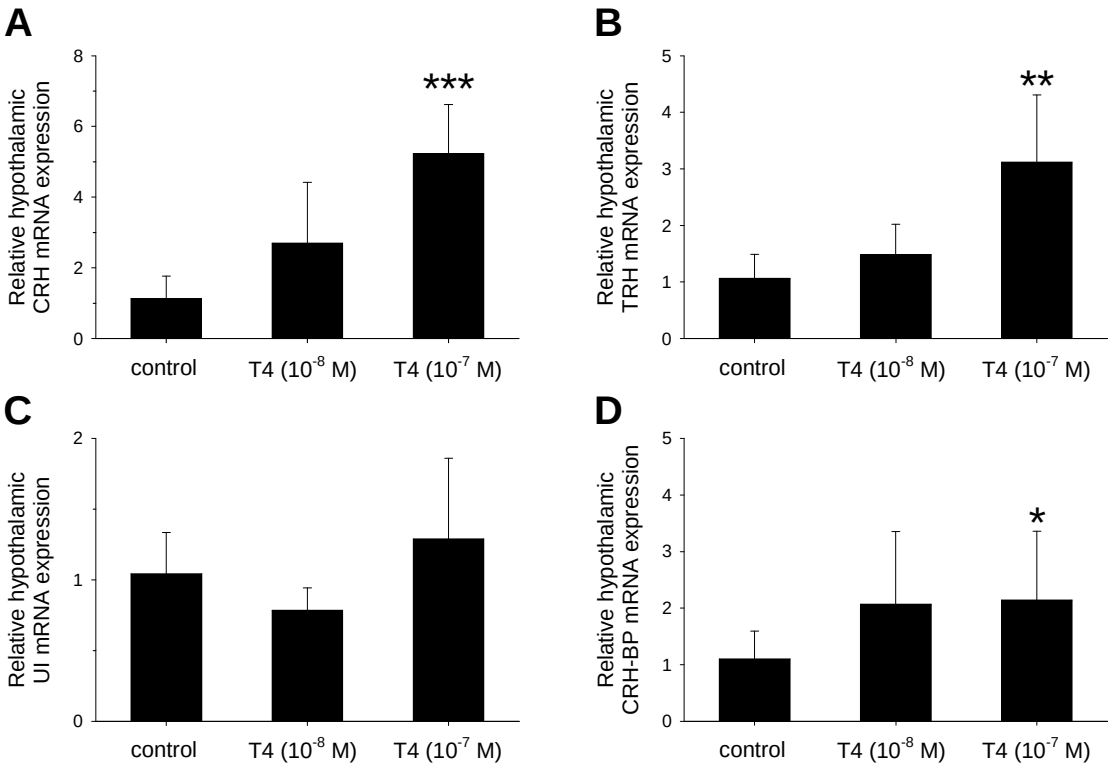

Fig 2. Relative mRNA expression levels of (**A**) CRH, (**B**) prepro-TRH, (**C**) UI and (**D**) CRH-BP in hypothalamic tissue (n = 6) incubated for 36 h with 10^{-8} M and 10^{-7} M T4.

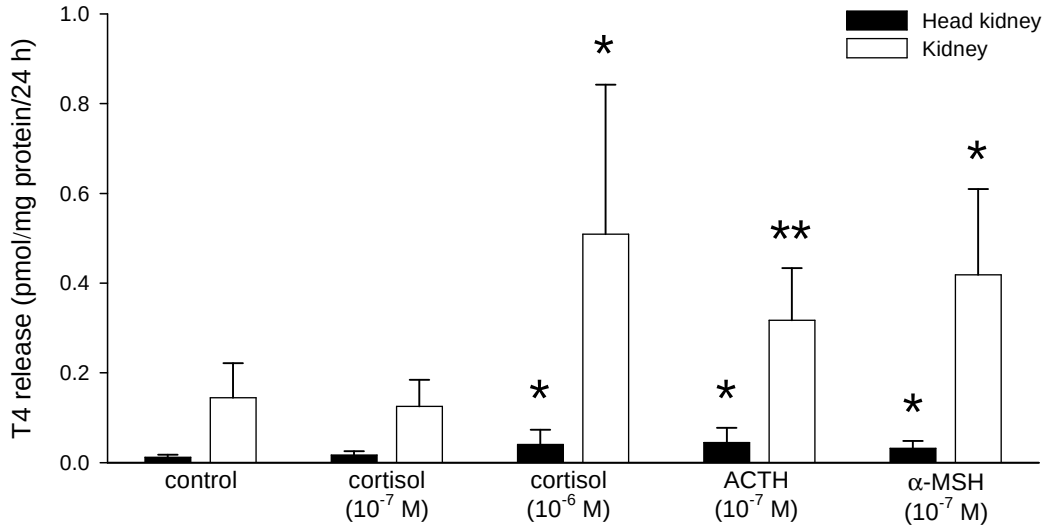

Fig 3. Release of T4 from head kidney and kidney tissue (n = 6-7) incubated for 24 h with cortisol (10^{-7} and 10^{-6} M), ACTH (10^{-7} M) and α-MSH (10^{-7} M, n = 3).

tissue 2- to 4-fold (Fig. 3). Basal and stimulated T4-secretion from the head kidney, overall, was 10 times lower than that of the kidney (Fig. 3).

Discussion

We demonstrate here that the NPO and renal tissues are putative sites for interaction between mediators of the HPT and the HPI axis in carp. Thyroxine affects the CRH system in the preoptic area. Peripherally, cortisol and ACTH both stimulate the release of thyroid hormones from renal tissues, *viz*. the head kidney and kidney.

Despite indications that preoptic CRH may be involved in the regulation of the HPT axis in some fish (De Groef, et al. 2006; Geven, et al. 2006; Kagabu, et al. 1998), no effect of CRH on the expression of carp pituitary TSH-β subunit could be demonstrated *in vitro*. The absence of a thyrotropic action of preoptic CRH can also be inferred from experimental results obtained *in vivo*. When carp were exposed to a 24 hr confinement stressor, the HPI axis was markedly activated, as exemplified by an increased expression of CRH mRNA in the preoptic area (Huising, et al. 2004). However, this increase was not accompanied by increased expression of pituitary TSH-β subunit, which remained unaffected in these animals (Dr. J. R. Metz, personal communication). Similarly, TRH did not alter the expression of TSH-β subunit gene *in vitro*, corroborating the results of Kagabu and colleagues (1998).

Taken together, these data provide no evidence for CRH and TRH as a hypothalamic thyrotropic factor in carp. However, the thyroid-stimulating properties of ACTH and α-MSH in carp may still confer a thyrotropic action to CRH and TRH. The present study focused on TSH as the pituitary thyroid-stimulating factor in carp (Geven, et al. 2006), but the identification of ACTH and α-MSH as two new putative pituitary thyroid-stimulating factors re-establishes CRH and TRH as potential hypothalamic thyrotropic factors, since the release of ACTH and α-MSH in carp is stimulated by CRH and TRH (Metz, et al. 2004; van den Burg, et al. 2003; van den Burg, et al. 2005). The identification of more than one hypothalamic thyrotropic and pituitary thyroid-stimulating factor in carp, clearly points to an integration of multiple endocrine signals for the control of the thyroid gland activity in teleostean fishes.

Thyroid hormones can modulate, at a central level, the HPI axis in carp. The expression of preoptic CRH mRNA is markedly increased upon exposure to T4 *in vitro*, although this effect could not be measured *in vivo* in hyperthyroid carp that were treated with T4. Here, the repeated injection of T4 produced a pronounced down regulation of the HPI axis as evidenced by a decreased level of plasma cortisol. The expression of preoptic CRH mRNA however, remained unaffected (Geven, et al. 2006). *In situ*, the preoptic CRH neuron is controlled by a multitude of stimulatory and inhibitory signals (Itoi, et al. 1998; Pisarska, et al. 2001). The different expression of CRH upon T4 exposure *in vitro* and *in vivo* which we observed can be explained by the fact that the denervated preoptic area *in vitro* does not receive efferent inhibitory signals, *viz*. glucocorticoids, norepinephrine, GABA, β-endorphin, dynorphin, somatostatin, galanin and substance P (*ibid*.). Our results indicate that the control of the preoptic CRH-neurons by T4 is modulated by other factors.

A consistent effect of T4 on the CRH system in carp is the stimulation of the expression of CRH-BP mRNA *in vitro* as well as in hyperthyroid carp *in vivo* (Geven, et al. 2006). Hypothalamic CRH-BP binds CRH (and UI) with a higher affinity than the type 1 CRH receptor, which reduces the bioavailability of CRH and, subsequently, the CRH-induced release of pituitary ACTH (Cortright, et al. 1995; Potter, et al. 1991; Westphal and Seasholtz 2006). Also in carp CRH-BP appears to be a functional modulator of hypophysiotropic CRH, since the expression of preoptic area CRH-BP mRNA is elevated upon a 24 h restraint stressor and CRH-BP is colocalised in CRH immunoreactive neurons projecting from the hypothalamus to pituitary corticotropes (Huising, et al. 2004). The extent to which the T4-induced increase in CRH-BP mRNA expression levels *in vitro* translates into increased functional protein concentrations in the hypothalamus *in situ* is difficult to estimate and awaits the development of a quantitative assay for CRH-BP protein. Still, our data suggest that CRH-BP from the preoptic area, where expression is T4-sensitive *in vitro* as well as *in vivo*, may fulfil a role as a central messenger that allows for the interaction of the HPT axis with the HPI axis in carp.

We also identified a peripheral site for the interaction between the HPT and the HPI axis: the head kidney and kidney. We already established that short- and long-term incubation with thyroid hormones have no effect on the

release of cortisol from head kidney fragments (Geven, et al. 2006). Conversely, exposure of head kidney and kidney fragments to cortisol and ACTH stimulated the release of T4 from these tissues. The thyroid hormone-releasing properties of cortisol and ACTH are consistent with the expression of their specific receptors, *i.e.* the glucocorticoid receptor and the type 2 melanocortin receptor respectively, in head kidney as well as kidney (Metz, et al. 2005; Stolte, et al. 2008).

Although cortisol has been shown to stimulate iodide uptake and thyroglobulin synthesis in synergy with TSH in several mammalian thyroid cell cultures (Becks, et al. 1992; Gérard, et al. 1989; Roger and Dumont 1983; Takiyama, et al. 1994), we here describe a direct and independent effect of cortisol on the thyroid gland of a teleost. Since the kidney is devoid of interrenal cells and a local cortisol-mediated effect therefore is not possible, we conclude that ACTH has a direct effect on the release of T4 in the kidney. Such a direct effect of ACTH could also apply to the head kidney. However, the ACTH-induced release of T4 may also represent a paracrine effect of endogenous cortisol released upon stimulation by ACTH, as exogenous cortisol mimicked the effect of ACTH on the release of T4 from head kidney tissue. Studies into the cellular localisation of the type 2 melanocortin receptor in head kidney tissue may reveal the presence of this receptor in thyrocytes, and thus can provide evidence for a direct mode of action of ACTH.

Another novel finding of this study is that the pituitary POMC-derived hormone α-MSH also has thyroid-stimulating properties in carp. The effect of α-MSH in the kidney is consistent with the expression of type 5 melanocortin receptor (Metz, et al. 2005). Although the MC5R is not expressed in the head kidney of carp, the thyroid-stimulating effect of α-MSH may be mediated by other melanocortin receptors, for instance, expression of the type 4 melanocortin receptor has been reported in the head kidney of rainbow trout and Japanese pufferfish (*Takifugu rubripes*) (Haitina, et al. 2004; Klovins, et al. 2004). In rat, binding of a specific analog for α-MSH was observed in the thyroid gland, indicating a regulatory role for α-MSH on thyroid gland metabolism in mammals (Tatro and Reichlin 1987).

We have found that thyroid hormones stimulate the release of α-MSH in carp *in vivo*. Hyperthyroid carp have plasma levels of α-MSH that are

increased by 30%, which are accompanied by increased mRNA expression levels of pituitary pars distalis POMC and PC1 (Geven, et al. 2006). In teleost fish, including carp, the release of pituitary α-MSH is mainly attributed to TRH (Lamers, et al. 1994; van den Burg, et al. 2003; van den Burg, et al. 2005). The *in vitro* stimulation of TRH by T4, and the *in vitro* stimulation of POMC and PC1 by TRH observed in this study, is commensurate to the endocrine cascade by which thyroid hormones control the release of α-MSH in carp.

The widespread distribution of melanocortin receptors in fish illustrates the pleiotropic functions for α-MSH, which includes the regulation of food intake and metabolism (Metz, et al. 2006b). The intracerebroventricular injection of [Nle^4, D-Phe^7]-α-MSH, an α-MSH agonist, inhibited food intake in goldfish (*Carassius auratus*) (Cerdá-Reverter, et al. 2003) and peripherally, α-MSH exhibited lipolytic effects in hepatocytes of rainbow trout (*Oncorhynchus mykiss*) (Yada, et al. 2000; Yada, et al. 2002). The concerted actions of thyroid hormones, cortisol and α-MSH on the regulation of metabolic processes may form the basis for the integration of these signals in carp.

In conclusion, this study identifies a central and a peripheral site in carp for the communication between the interrenal axis and the thyroid axis and, additionally, α-MSH as a thyroid-stimulating factor. Centrally, T4 can inhibit the HPI axis via CRH-BP in the preoptic area. Peripherally, cortisol and ACTH stimulate the release of T4 from renal tissues, as does α-MSH. The intimate interrelationships between these neuroendocrine systems are pivotal for the regulation of general metabolism.

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Chapter 6

Dithiothreitol requirements and enzyme kinetics of renal and hepatic iodothyronine 5'-deiodinase activity in common carp (*Cyprinus carpio* L.)

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Abstract

Iodothyronine deiodinases determine the biological activity of thyroid hormones. Despite a high homology of the catalytic sites of mammalian and teleostean deiodinases, requirements for the putative thiol co-substrate DTT vary considerably between fish species. To further our insights in the interactions between the deiodinase protein and iodothyronine and DTT, we measured enzymatic iodothyronine 5'-deiodination, *Dio1* and *Dio2* mRNA expression, and affinity probe binding in liver and kidney preparations from a freshwater teleost, the common carp (*Cyprinus carpio* L.). Deiodination rates were analysed as a function of the iodothyronine and DTT concentrations. In kidney rT3 5'-deiodinase activity is best described by two-site enzyme kinetics with a high- and low-affinity component. The low-affinity component is here completely inhibited by 1 mM DTT. In contrast, rT3 5'-deiodination in liver is potently stimulated by 1 mM DTT. The different biochemical characteristics of 5'-deiodination in liver and kidney are not associated with a single enzymatic entity as the expression of both *Dio1* and *Dio2* mRNA was demonstrated in both organs. In liver and kidney, DTT stimulates the incorporation of *N*-bromo-acetylated affinity labels in proteins with estimated molecular masses of 57 and 55, and 31 and 28×10^3, respectively. Paradoxically, in tissues in which *Dio1* and *Dio2* mRNA expression was not detectable we did measured 5'-deiodination enzyme activities and affinity label incorporation by two distinct protein species. A general model for 5'-deiodination in vertebrates cannot, as yet, be constructed. Despite the homologies in primary structure, the biochemistry of fish deiodinases differs markedly from their mammalian counterparts.

Introduction

The biological genomic actions of thyroid hormones are attributable to 3,5,3′-tri-iodothyronine (T3) that is derived from thyroxine (T4) via enzymatic deiodination in extrathyroidal tissues. Iodothyronine deiodinases, a class of selenoenzymes, catalyze the deiodination of the phenolic and tyrosyl ring of the thyroid hormone molecule. Specifically, it is the monodeiodination of the phenolic ring, also termed 5′-deiodination or outer-ring deiodination, that generates the bioactive thyroid hormone T3 from the prohormone T4. In vertebrate species, from fishes to humans, deiodinases type 1 and type 2 are both capable of 5′-deiodination (Bianco, et al. 2002; Köhrle 2000; Orozco and Valverde-R 2005), and are thus important determinants of circulating thyroid hormone levels (Darras, et al. 1998; de Jong, et al. 2007; Van der Geyten, et al. 1998) and of the availability of intracellular T3 that can activate nuclear thyroid hormone receptors (Köhrle 2000).

Their pivotal role in thyroid hormone metabolism makes deiodinases key regulators of physiological processes that are steered by thyroid hormones. Classic examples are mammalian ontogenetic development and amphibian metamorphosis (Galton 2005; Morvan Dubois, et al. 2006; Van der Geyten, et al. 2007). Similarly, metamorphosis and, in analogy, parr-smolt transformation in fishes are initiated and controlled by thyroid hormones (Ebbesson, et al. 2000; Inui and Miwa 1985; Klaren, et al. 2008; Miwa and Inui 1987; Sweeting, et al. 1994), although the role of deiodinases is not as well investigated as in amphibians and mammals. Other physiological processes controlled by thyroid hormones, such as osmoregulation (Arjona, et al. 2008; Klaren, et al. 2007; López-Bojorquez, et al. 2007; Orozco, et al. 2002) and energy metabolism (de Pedro, et al. 2003; Finnson and Eales 1999; Power, et al. 2000; Van der Geyten, et al. 1998), are regulated *via* deiodinases, often in concert with other hormones (Geven, et al. 2006; Leloup and Lebel 1993; Shrimpton and McCormick 1998). The expression of deiodinases, and in particular their 5′-deiodination activities are therefore valuable experimental readouts in clinical and physiological studies.

All mammalian deiodinases studied to date require the presence of a thiol compound *in vitro*. The sulfhydryl reagent dithiothreitol (DTT) is generally used, but the endogenous thiol compound has still not been unequivocally

identified. In contrast to what we know for mammals, the *in vitro* requirements for DTT of deiodinases in a number of teleost species vary considerably. Indeed, inhibitory and stimulatory effects on enzymatic deiodination have been reported (Arjona, et al. 2008; Klaren, et al. 2005; Mol, et al. 1997; Orozco, et al. 2000; Orozco, et al. 1997). The deduced amino acid sequences of vertebrate deiodinases, fishes included, contain highly homologous catalytic sites (Kuiper, et al. 2005) and conserved cysteine residues that are involved in the interactions with thiols (Croteau, et al. 1998; Klaren, et al. 2005). It is not possible yet to predict the effect of DTT on enzymatic deiodination based on a primary deiodinase amino acid sequence alone. Consequently, we cannot assume general similar thiol requirements for vertebrate deiodinases in *in vitro* assays.

In a series of experiments we established stimulatory and inhibitory actions of DTT on the enzymatic 5'-deiodination of reverse T3 (3,3',5'-triiodothyronine), a preferred substrate of mammalian deiodinase type 1, in liver and kidney from common carp (*Cyprinus carpio* L.). To further our insights in the interactions between the deiodinase enzyme and its substrates, deiodination rates were analysed as a function of, respectively, iodothyronine and DTT concentration. The expression of *Dio1* and *Dio2* mRNA and DTT-requirements of 5'-deiodination activities in different tissues were assessed. The interactions between enzyme and substrates were further investigated using bromoacetylated iodothyronine affinity probes and polyacrylamide gel electrophoresis.

Materials and methods

Animals and animal procedures

Common carp (*Cyprinus carpio* L.), hereafter called carp, were from laboratory stock. Fish were kept in 150-l tanks with recirculating filtered Nijmegen city tap water at 22°C, at a photoperiod of 16 hours light and 8 hours of darkness. Fish were fed a commercial fish food (Trouvit, from Trouw Nutrition International, Putten, The Netherlands) at a ration of 1.5% of their estimated body weight per day. Animals were deeply anesthetized with 0.1% 2-phenoxyethanol and killed by spinal transection. All animal procedures were approved by the local ethical review committee.

Materials

Reverse T3 (rT3, 3,3',5'-triiodo-L-thyronine), T3 (3,5,3'-triiodo-L-thyronine), and T4 (3,5,3',5'-tetraiodo-L-thyronine) were purchased from Sigma Chemical Co. (St. Louis, MO). Dithiothreitol (DTT) was obtained from Biotech Trade & Service (St. Leon-Rot, Germany). Solutions of DTT in water were prepared freshly before experimentation, and contained no oxidized DTT as determined by the absence of a disulfide absorbance peak at 283 nm in the ultraviolet spectrum (Cleland 1964). Sephadex LH-20 was from Amersham Pharmacia Biotech (Uppsala, Sweden). Radioactively labeled [^{125}I]rT3 (27.8-46.3 MBq/μg), [^{125}I]T4 (40-48.8 MBq/μg), and [^{125}I]T3 (40-48.8 MBq/μg) were from NEN Life Science Products, Inc. (Boston, MA). All other chemicals were analytical grade and obtained from commercial suppliers.

Tissue homogenates

Liver and kidney from carp were homogenized in phosphate buffer (100 mM Na-phosphate, 2 mM EDTA, pH 7.2) in a glass dounce homogenizer equipped with a tightly fitting Teflon pestle operated manually. Homogenates were stored at -20°C until further analysis. Protein was measured with a commercial Coomassie Brilliant Blue reagent kit (Biorad, München, Germany) and bovine serum albumin (BSA) as reference.

Iodothyronine 5'-deiodinase assays

5'-Deiodinase activities were assayed in duplicate by incubation of 50 μg homogenate protein for 15 min at 37°C in 200 μl 100 mM phosphate/2 mM EDTA buffer (pH 7.2) as described earlier (Klaren, et al. 2005). Iodothyronines and DTT were varied in concentrations as indicated in the legends to the figures. Radiotracer was purified on a 10% (w/v) Sephadex LH-20 mini-column shortly before use, and was added to a nominal amount of 1×10^5 cpm per reaction. This yielded a specific activity of 9×10^{13} to 2×10^{15} cpm/mol iodothyronine in the final incubation medium. The contribution of the tracer to the total tracee concentration was always less than 0.5%. Total iodothyronine

concentrations in the medium were strictly determined by the unlabeled iodothyronine. The incubation was quenched by the addition of 100 µl 5% w/v ice-cold BSA, followed by 500 µl 10% w/v ice-cold trichloroacetic acid and precipitation of denatured proteins at 1400 × *g* (15 min, 4°C). Liberated iodide was isolated from the incubate with the use of Sephadex LH-20 column chromatography, collecting $^{125}I^-$ in the first two 1-ml 0.1 M HCl eluates. Radioactivity was measured in an LKB-1272 Clinigamma gamma counter (Wallac Oy, Turku, Finland). The specific [^{125}I]iodothyronine activity in the incubation medium was used to express the deiodination rate as fmol total iodothyronine deiodinated per min per µg protein. Our calculations of the specific rT3 and T4 deiodination rates included a correction factor of 2 to take into account the random labeling of the 3'- and 5'-positions of [^{125}I]rT3 and [^{125}I]T4. Deiodination rates were corrected for non-enzymatic 5'-deiodination measured in the absence of tissue homogenate.

Identification of carp Dio1 and cloning and sequencing of Dio2 cDNA

Total RNA was extracted from selected tissues using the TRIzol reagents (Invitrogen, Carlsbad, CA). Following treatment with DNase, 1 µg of RNA was reverse-transcribed to cDNA in a 20-µl reaction mixture containing 300 ng random primers, 0.5 mM dNTPs, 10 mM dithiothreitol, 10 U RNase Inhibitor and 200 U Superscript II Reverse Transcriptase (Invitrogen) for 50 min at 42°C. The carp *Dio1* sequence was reconstructed from two ESTs (GenBank accession numbers: CA966709, CA967152) that were obtained from a transcript screening study (Gracey, et al. 2004). To retrieve the carp *Dio2* sequence, degenerated primers (obtained from Biolegio BV, Nijmegen, The Netherlands) were designed based on conserved sequences after aligning the *Dio2* sequences of puffer fish (*Takifugu rubripes*, GenBank accession number AB360768), zebrafish (*Danio rerio*, NM212789) and mouse (NM010050). A polymerase chain reaction (PCR) was carried out on carp kidney cDNA using the degenerated primers. PCR products were separated by electrophoresis on a 1% agarose/ethidium bromide gel, ligated into a pCR4-TOPO vector, and introduced into competent TOP10 *E. coli* cells (TOPO TA Cloning kit, Invitrogen). Successfully transfected plasmids were extracted using a Miniprep kit (Biorad, Hercules, CA),

sequenced using the dideoxy method of Sanger, et al. (1977) as applied in the BigDye® Terminator v.1.1 Cycle Sequencing kit (Applied Biosystems Benelux, Nieuwerkerk aan den IJssel, The Netherlands) and analyzed on an ABI Prism 310 automated sequencer (Applied Biosystems). The 5'- and the 3'-cDNA termini were obtained by rapid amplification of cDNA ends (RACE)-PCR using the GeneRacer protocol (Invitrogen). RACE-PCR products were sequenced as described above. Two overlapping partial carp *Dio2* nucleotide sequences, *Dio2.1* and *Dio2.2*, were obtained. The deduced amino acid sequences of both cDNAs contained a consensus catalytic site with a selenocysteine, aligned well and clustered with other vertebrate *Dio2* sequences.

Dio1 and Dio2 tissue distribution

Selected organs and tissues were removed, snap frozen on dry ice and stored at -80°C. Total RNA was extracted and transcribed to cDNA as previously described. Gene expression of *Dio1* and *Dio2* was assessed by RT-PCR using specific primers. Briefly, a 20-µl reaction mixture, consisting of 1 µl cDNA template, 2 µl 10 × PCR buffer, 0.6 µl $MgCl_2$ (50 mM), 0.3 µl dNTP mix (25 mM), 1 µl forward and reverse primer (10 µM), 14 µl H_2O and 0.1 µl Taq DNA polymerase (Invitrogen) was subjected to 40 cycli of 94°C for 60 s, 60°C for 60 s and 72°C for 60 s, followed by a final extension step at 72°C for 10 minutes. PCR products were analyzed by electrophoresis on a 1.5% agarose gel.

Real-time quantitative PCR

The expression of *Dio1* and *Dio2* mRNA relative to the expression of 40S ribosomal protein S11 (GenBank accession number AB012087) was measured by use of real-time quantitative PCR (RQ-PCR) and specific primers for *Dio1*, *Dio2* and 40S. Primers for *Dio2* were designed to amplify sequences from both *Dio2.1* and *Dio2.2* cDNAs. In general, cDNA was diluted 50-fold, 5 µl cDNA was used in a 25 µl reaction mixture consisting of 12.5 µl SYBR Green Master Mix (Applied Biosystems) and 3 µl of each primer (600 nM final concentration) was added. RQ-PCR was performed on a GeneAmp 5700 Sequence Detection System (Applied Biosystems). The reaction mixture was incubated for 10 min at

95°C, followed by 40 cycles of 15 s denaturation at 95°C and 1 min annealing and extension at 60°C. Melting curves that yielded single peaks confirmed the identity and specificity of the PCR reactions. Dissociation plots were analyzed and cycle threshold (CT) values were determined.

Synthesis of N- bromoacetylated probes and affinity labeling

N-BrAc-iodothyronines were synthesized from [^{125}I]T4 and [^{125}I]T3, respectively, and bromoacetylchloride in dry ethyl acetate as described by Mol, et al. (1984). The product was separated from the incubate by using a Sephadex LH-20 column, equilibrated with 0.1 M HCl, by elution with absolute ethanol. The purity of the probe was verified using thin layer chromatography and nuclear magnetic resonance spectroscopy. The solvent was evaporated under a stream of nitrogen shortly before use, and the radioprobe was added to an amount of 2×10^5 cpm per reaction. One hundred μg of homogenate protein was incubated for 30 min at 37°C in a volume of 50 μl 100 mM phosphate/2 mM EDTA buffer (pH 7.2) to which 1 mM DTT was added as indicated in the figures. These incubation conditions are similar to those of the iodothyronine deiodinase assay. The reaction was quenched by the addition of 50 μl SDS-PAGE loading buffer and heated at 95°C for 5 min. Twenty μl of the quenched incubate was separated on a 12% polyacrylamide gel according to the method of Laemmli (1970). Gels were fixated, and autoradiography was performed by exposure to Kodak Biomax MR film at room temperature.

Analysis and statistics

All data are presented as mean values ± standard error of the mean (SEM), unless stated otherwise. The number of different preparations (*n*) is given in parentheses. Kinetic data were fitted to the rate equations given in the legends to the figures. We used the non-linear regression data analysis program EnzFitter v. 2.0.18.0 (Biosoft, Cambridge, UK) that employs the Levenberg-Marquardt algorithm for weighted least squares estimation of parameters. In addition, the program Leonora v. 1.0 (Cornish-Bowden 1995a), which allows a wider choice of curve-fitting methods and weighting algorithms, was used.

Since the calculation results of both programs were in close agreement, we only show those obtained with EnzFitter. Parameters values are given with their 95% confidence limits in the legends to the figures. Statistical significance was evaluated with Student's *t*-test, and was accepted at $P < 0.05$.

Results

Renal deiodination

DTT potently inhibits 5′-deiodination of T4, T3 and rT3 by carp kidney homogenates (Fig. 1A). At the lowest DTT concentration tested, *viz.* 0.5 mM, 5′-deiodination rates are already inhibited by 65 to 73%. The inhibitory action of DTT in carp kidney is reflected in the 35-fold lower V_{max} of rT3 5′-deiodination measured in the presence of 1 mM DTT (Fig. 1B). Analyzed as a function of the rT3 concentration, initial rates measured in the absence of DTT are described by a double Michaelis-Menten equation, as visualized by the non-linearity of the Eadie-Hofstee transformation (Fig. 1B). Measured in the absence of DTT, the calculated values for the kinetic parameters V'_{max} and K'_M of the low-affinity 5′-deiodination activity are 597 fmol/min/μg and 11 μM rT3, respectively. For the high-affinity component we calculate: V''_{max} = 16 fmol/min/μg, and K''_M = 1.6 μM rT3. In the presence of 1 mM DTT, the inhibited renal rT3 deiodination rates obey simple, single-site Michaelis-Menten kinetics described by a V_{max} of 17 fmol/min/μg protein, and a K_M of 1.0 μM rT3 (Fig. 1B). These values are similar to those of the high-affinity deiodination component measured in the absence of DTT.

Hepatic deiodination

DTT dose-dependently stimulates 5′-deiodination in carp liver homogenates. Figure 2A shows that the stimulatory effect of DTT on 5′-deiodination in carp liver homogenates saturates at DTT concentrations ≥ 0.5 mM. Maximum deiodination rates for T3 and T4 were already measured at the lowest DTT concentration tested, *viz.* 0.5 mM. The DTT dose-dependency of rT3 deiodination is described by a Michaelis-Menten equation to which a constant

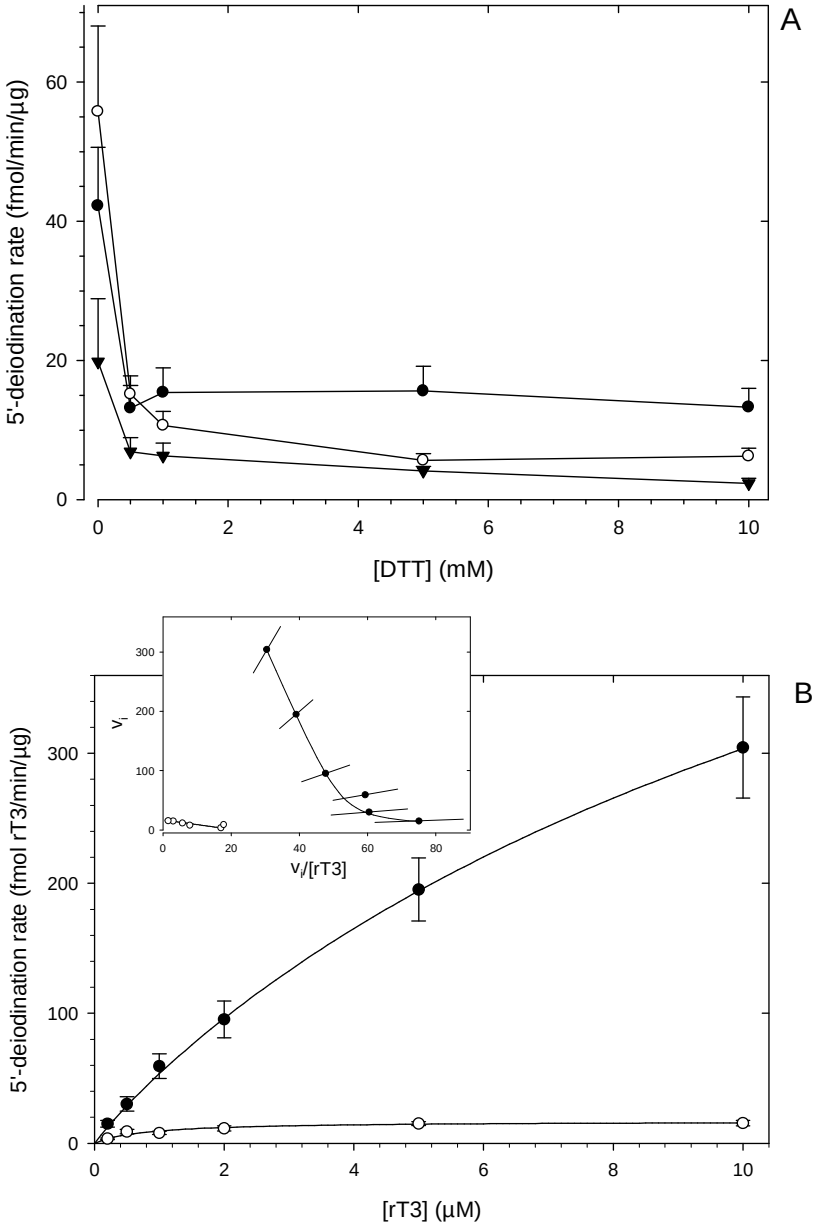

Figure 1. Iodothyronine 5′-deiodination rates in carp kidney homogenates. **A.** Effects of DTT on rT3 (●), T4 (○) and T3 (▼) 5′-deiodination (n = 4). Measurements of initial rates were in the presence of 5 µM iodothyronine. **B.** rT3 5′-deiodination, measured at initial rate (v_i), as a function of substrate concentration (n = 4). DTT was either omitted (●) from the incubation medium or present at 1 mM (○). In the absence of DTT, data points are described by a double Michaelis-Menten rate equation: $v_i = \frac{V'_{max} \times [S]}{K'_M + [S]} + \frac{V''_{max} \times [S]}{K''_M + [S]}$ with parameter values: V'_{max} = 597 (568 – 625), V''_{max} = 16 (5 – 28) fmol/min/µg; K'_M = 11 (10.5 – 12.1), K''_M = 1.6 (-2.1 – 5.3) µM rT3. Deiodination rates measured in the presence of 1 mM DTT are adequately described by a single Michaelis-Menten equation: V_{max} = 17.3 (17.29 – 17.37) fmol/min/µg protein, and K_M = 1.04 (1.03 – 1.05) µM rT3. The insert shows an Eadie-Hofstee transformation (v_i *vs.* v_i/[rT3]) of the data points and calculated kinetic parameters.

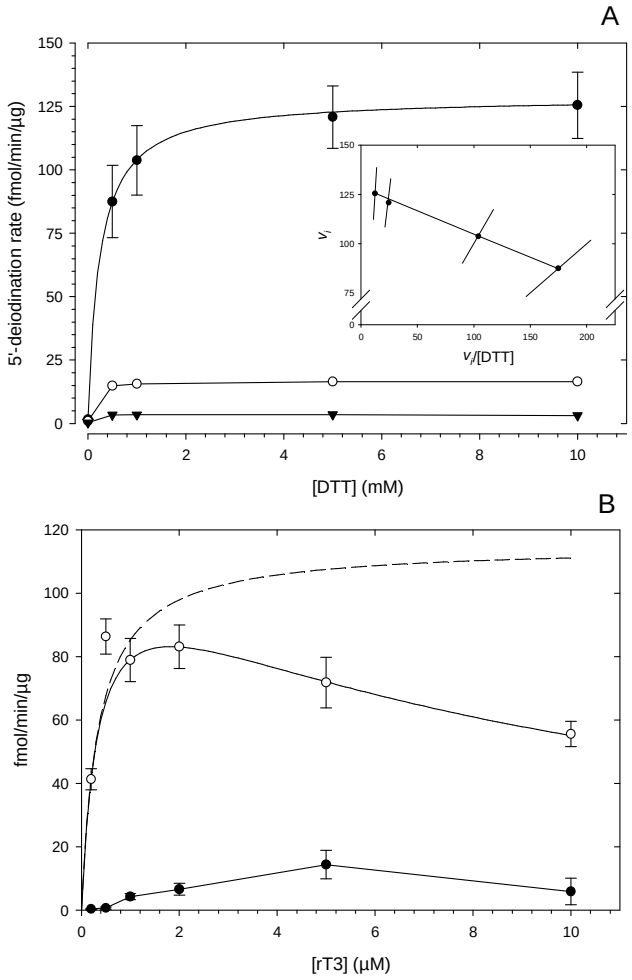

Figure 2. Iodothyronine 5′-deiodination rates in carp liver homogenates. **A.** Effects of DTT on rT3 (●), T4 (○) and T3 (▼) 5′-deiodination rates (n = 4). Measurements were in the presence of 5 µM iodothyronine. For T4 and T3, error bars are smaller than the symbols used. Reverse T3 5′-deiodination is described by a modified Michaelis-Menten equation: $v_i = \frac{V_{max} \times [S]}{K_M + [S]} + c$, in which constant c represents 5′-deiodination measured in the absence of DTT, *i.e.* 1.6 ± 0.1 fmol/min/µg protein. Calculated kinetic parameters are: V_{max} = 127 (125 – 128) fmol/min/µg protein, K_M = 0.24 (0.22 – 0.25) mM DTT. The inset shows an Eadie-Hofstee transformation (v_i *vs.* v_i/[DTT]) of the data and calculated kinetic parameters. **B.** Reverse T3 5′-deiodination, measured at initial rate (v_i), as a function of substrate concentration (n = 6 – 7). DTT was omitted (●) from the incubation medium or present at 1 mM (○). Initial rates measured in the presence of DTT are fitted to a rate equation describing excess substrate inhibition: $v_i = \frac{V \times [S]}{K_M + [S] + [S]^2 / K_{si}}$ (Cornish-Bowden 1995b). Calculated parameters are: apparent limiting rate V = 115 (111-119) fmol/min/µg protein, apparent Michaelis constant K_M = 0.35 (0.31 – 0.40) µM rT3, and substrate inhibition constant K_{si} = 9.5 (8.5 – 10.5) µM rT3 in the presence of 1 mM DTT, and are indicated by the solid curve. The dashed line is calculated from the Michaelis-Menten equation with V_{max} = 115 fmol/min/µg protein, K_M = 0.35 µM rT3, with no substrate inhibition. Data points measured in the absence of DTT did not converge satisfactorily to either of these rate equations.

term *c* was added to formally allow for the deiodination measured in the absence of DTT. The limiting rate V_{max} is calculated to be 127 fmol/min/μg protein, the Michaelis constant K_M 0.2 mM DTT. Constant *c* was measured to be 1.6 fmol/min/μg protein, or a negligible 1.3% of the calculated V_{max} value. Data points and calculated parameters of hepatic DTT-dependent rT3 5′-deiodination converge on a linear Eadie-Hofstee plot (Fig. 2A), which is indicative for a single catalytic component. Analyzed as a function of the rT3 concentration, 5′-deiodination rates in liver homogenates measured in the presence of 1 mM DTT are one to two orders of magnitude higher than in the absence of DTT (Fig. 2B). Deiodination rates stimulated by DTT reach a maximum with increasing rT3 concentrations and then decrease. This pattern is well described by a rate equation describing excess substrate inhibition (see the legend to Fig. 2B). Data points measured in the absence of DTT show a trend towards excess substrate inhibition, but are not adequately described by the appropriate rate equation.

Dio1 and Dio2 mRNA expression and 5′-deiodination: tissue distribution

Figure 3 shows the expression of *Dio1* and *Dio2* mRNA in carp tissues. Both are expressed in liver and kidney. Furthermore, head kidney, the subpharyngeal region, testis, skeletal muscle, and pituitary gland also express *Dio1* and *Dio2*. Gut and ventricle exclusively express *Dio1*, gills express *Dio2* only. In the brain, both deiodinases are detected in the hypothalamus including the nucleus preopticus, but *Dio2* clearly is expressed in more brain areas than *Dio1* is. In organs that express either *Dio1* or *Dio2*, *i.e.* gills and ventricle, DTT inhibited rT3 5′-deiodination (results not shown). Quantitatively, *Dio2* expression levels in liver and kidney are higher than those of *Dio1* (Fig. 4). The expressions of *Dio1* and *Dio2* in the liver are 6- and 2.5-fold higher, respectively, than that in kidney. The results obtained thus far indicate that the differences in DTT-sensitivity and enzyme kinetics between rT3 5′-deiodination in kidney and liver are not reflected in the expression of *Dio1* and *Dio2* mRNA in these organs.

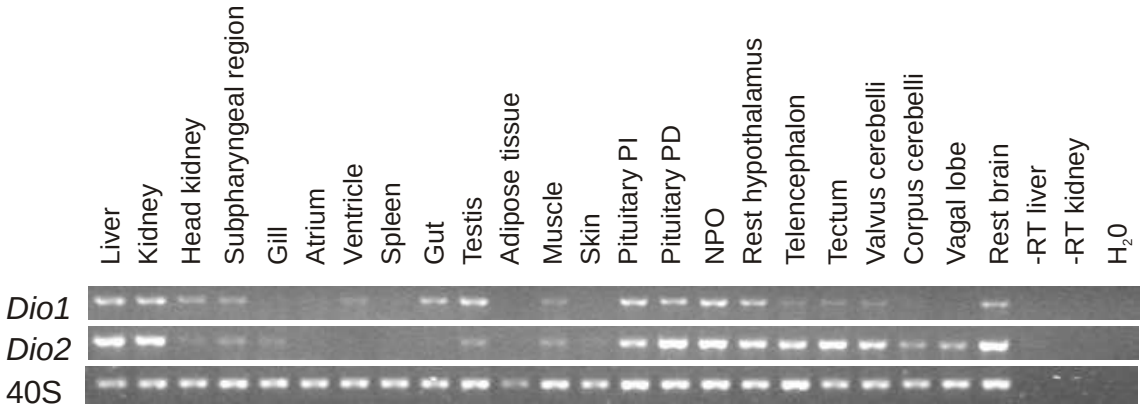

Figure 3. Expression of *Dio1*, *Dio2* and 40S mRNA in carp organs, as measured by RT-PCR. Control reactions were run in the absence of reverse transcriptase (-RT liver, -RT kidney) and in the absence of cDNA template (H_2O). Abbreviations: PI, pars intermedia; PD, pars distalis; NPO, nucleus preopticus.

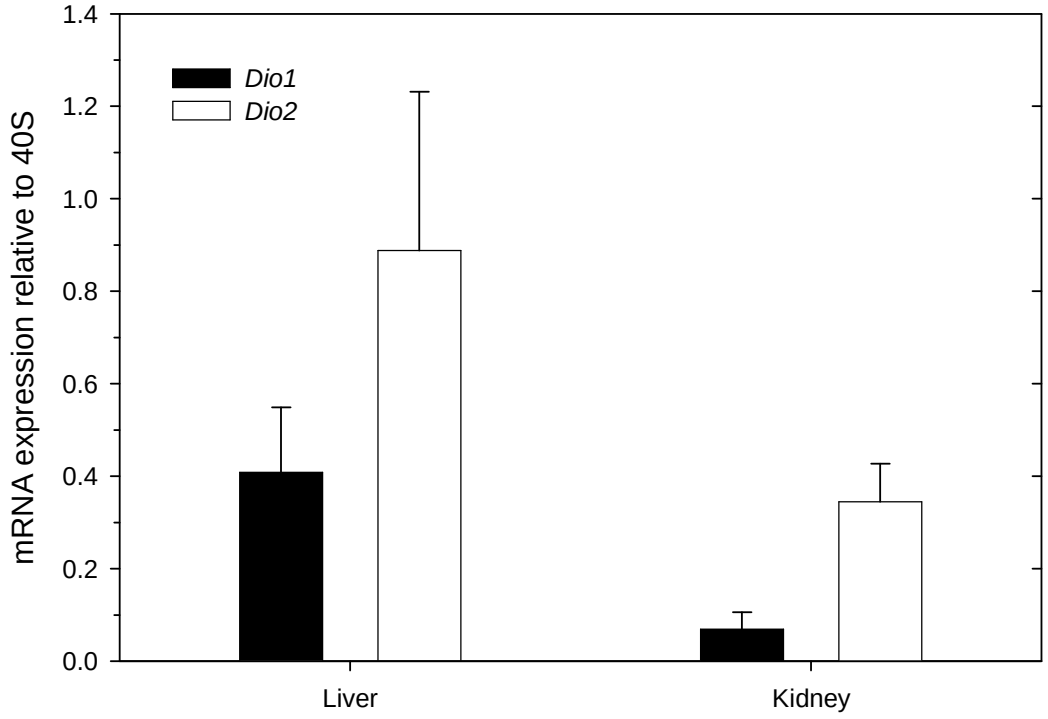

Figure 4. Expression levels of *Dio1* (closed bars) and *Dio2* (open bars) mRNA relative to 40S mRNA in carp liver and kidney (n = 16). Mean values + 1 SD are shown.

Affinity labelling

Figure 5 shows that the *N*-bromoacetylated affinity probes bind to two groups of proteins in carp kidney and liver homogenates. One group consists of two proteins with estimated relative molecular weights of 57×10^3 and 55×10^3. The second group consists of two smaller proteins with estimated molecular weights of 31×10^3 and 28×10^3. In liver homogenates, DTT enhances the binding of the affinity probes to the lower molecular weight proteins. This effect is absent in kidney, but here the labeling of the higher molecular weight proteins is enhanced in the presence of 1 mM DTT.

Discussion

The main conclusion of this investigation is that iodothyronine 5'-deiodination, measured *in vitro* in liver and kidney of the same animal species, have opposed requirements for dithiothreitol. Moreover, the substrate dependency of rT3 5'-deiodination typically is described by rate equations that are more complex than simple Michaelis-Menten kinetics. Neither liver nor kidney mutually exclusively expresses *Dio1* or *Dio2* mRNA, and thus the differences in the biochemistry of rT3 5'-deiodination cannot be attributed to an organ-specific expression of a particular deiodinase type. Intra- and inter-species differences in deiodinase activities warrant a careful and critical characterisation of 5'-deiodinase *in vitro* assays.

The response of enzymatic 5'-deiodinaton reactions to DTT *in vitro* in fish is species- and tissue-specific, variable, and comes in extremes. Often DTT increases the reaction rate (Mol, et al. 1998), but in a number of tissues from different teleost species it is without effect (Mol, et al. 1997; Orozco, et al. 1997) or even inhibitory (Arjona, et al. 2008; Klaren, et al. 2005; Orozco, et al. 2000). DTT plays a key role in the proposed catalytic mechanism of 5'-deiodination, where it reduces the selenyl-iodide intermediate to regenerate the native enzyme (Leonard and Visser 1986). The catalytic sites including selenocysteine of all deiodinases characterized to date are very well conserved (Kuiper, et al. 2005), as are the cysteine residues that are involved in the interactions with thiols (Croteau, et al. 1998; Klaren, et al. 2005). In particular in this respect the observed

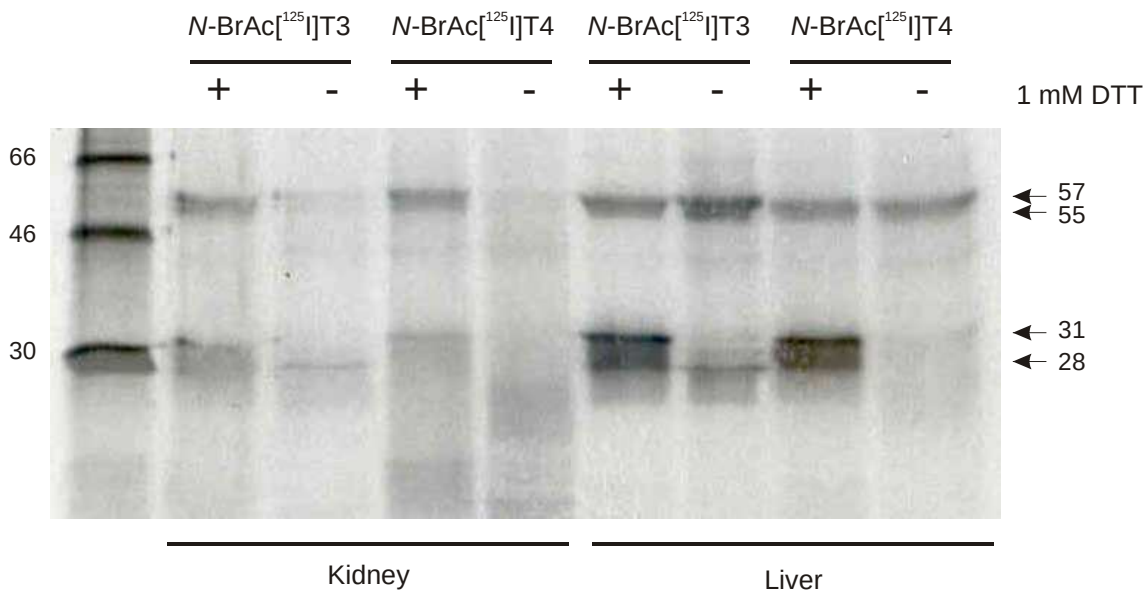

Figure 5. Binding of *N*-bromoacetylated ^{125}I-labeled T3 and T4 probes in carp kidney and liver homogenates fractionated by SDS polyacrylamide gel electrophoresis. Homogenates were incubated in the presence or absence of 1 mM DTT, indicated by '+' and '-', respectively. Relative molecular weights (×10^3) of the ^{14}C-labeled markers (left hand lane), and those of the labeled proteins are indicated.

opposed *in vitro* requirements for DTT of vertebrate deiodinases remain enigmatic. Still, it is clear that not all deiodinases require the presence of a thiol-containing compound to catalyze the deiodination of iodothyronines.

5'-deiodination activity in carp kidney is composed of two components. The calculated Michaelis constant K_M of the DTT-resistant high-affinity component, 1.6 μM rT3, corresponds well with those of rT3 5'-deiodination measured in renal microsomal fractions isolated from four different teleost species as reported by Mol et al. (1998; 1997). However, these studies were all performed in the presence of high, *i.e.* 10 to 30 mM, concentrations DTT, even though it was shown that renal rT3 5'-deiodination in one of these fish species is not stimulated by DTT (Mol, et al. 1997). The low- and high-affinity components likely reflect two independently working enzymes in carp kidney. This is corroborated by the substantial expression of both *Dio1* and *Dio2* mRNA and the, albeit less pronounced, binding of T3- and T4-affinity labels by multiple protein species in this organ. DTT specifically inhibits the low-affinity (K_M 11

μM rT3) 5′-deiodination component in carp kidney. The optimum rT3 5′-deiodination activity, *i.e.* measured in the absence of DTT in carp kidney, only marginally saturated at the highest rT3 concentration tested (*viz.* 10 μM), and thus has an overall total affinity that is several orders of magnitude lower compared to other vertebrate species. The inclusion, traditionally, of millimolar amounts of DTT in incubation media could very well mask DTT-sensitive components in whole-organ 5′-deiodination, and explain the single-site enzyme kinetics and low Michaelis constants reported (Mol, et al. 1998).

The inhibition of 5′-deiodination at high rT3 concentrations that we observed in carp liver homogenates is considered to be positively diagnostic for a two-substrate ping-pong mechanism (Cornish-Bowden 1995b), and thus hints at the involvement of a deiodinase type 1 for which in mammals the same reaction mechanism is proposed (Goswami and Rosenberg 1984; Leonard and Visser 1986; Visser 1979). It is tempting to interpret the stimulatory action of DTT on hepatic 5′-deiodination in favour of a chemical mechanism in which DTT reduces the iodide-substituted deiodinase. In the simplest mechanism only one selenyl-iodide reduction is necessary to complete one 5′-deiodination catalytic cycle, and iodothyronine and DTT would thus react with a 1:1 stoichiometry. However, we here show that in carp liver the 5′-deiodination of 10 μM rT3 only saturates at DTT concentrations higher than 1 mM. The 100-fold difference between the saturating concentrations of the two substrates suggests a very different molecularity of 5′-deiodination, not only in carp liver, but also in other teleost and mammalian preparations where typically millimolar DTT and (sub)micromolar iodothyronine concentrations are used (Chopra 1978; Mol, et al. 1998).

In a series of preliminary experiments we were unsuccessful to spectrophotometrically detect the appearance of oxidized DTT during the time-course of 5′-deiodination catalyzed by rat liver microsomes, a well-characterized preparation, in the presence of 1 mM reduced DTT and 10 μM rT3 (results not shown). Oxidized DTT has a very low maximum molar absorption coefficient of 273 $M^{-1}cm^{-1}$ at 283 nm (Cleland 1964) which makes it virtually impossible to measure, using a standard 1-cm path length, the micromolar amounts that would be generated according to a 1:1 iodothyronine:DTT stoichiometry. Still, following the Beer-Lambert law, the oxidation of concentrated DTT solutions

gives substantial absorbances that we indeed detected when we allowed a 1-mM DTT solution to oxidize on air. The incubation of microsomes with 1 mM DTT and 10 μM rT3 did not result in detectable concentrations oxidized DTT (results not shown). These observations do not necessarily disprove a 1:1 stoichiometry of the 5'-deiodination reaction. They do indicate, however, that the larger fraction of DTT is not oxidized upon rT3 5'-deiodination by a mammalian deiodinase (that is stimulated by DTT), and thus appears not to be involved in the chemical deiodination reaction. We are currently further investigating the reaction mechanism by simultaneously measuring DTT oxidation and the liberation of radioiodide in the course of 5'-deiodination of an iodothyronine.

The presence of DTT increases the incorporation of *N*-bromoacetylated radioiodothyronines in different protein species in liver, kidney, and other peripheral tissues. The pattern of affinity labeling of liver proteins differs from that in kidney and other organs in that predominantly protein species with relative masses of 28×10^3 and 31×10^3 had incorporated *N*-BrAc-iodothyronines upon incubation with DTT. A similar effect of the DTT stereoisomer dithioerythritol (DTE) was found in rat liver microsomes by Köhrle, et al. (1990). The protein species detected by autoradiography hint at the presence of deiodinase types 1 and 2 monomers, although the covalent binding of the bromoacetylated probes is likely to interfere with the migration of proteins on a polyacrylamide gel and the estimation of molecular masses. The stimulatory effect of DTT in carp liver can, tentatively, be interpreted as an increased binding of substrate to the catalytic site of the enzyme, and not so much by the reduction of a putative substituted enzyme intermediate which would result in the loss of radioiodide from the substituted enzyme intermediate. The inhibitory action of DTT on renal 5'-deiodination cannot be explained by the prevention of the formation of an enzyme-substrate complex, in the kidney probably a deiodinase dimer. The emergence of protein-substrate complexes in renal homogenates in the presence of DTT (Fig. 8) that are potently inhibited by the same thiol (Fig. 1) hints at the generation of a catalytically inactive dead-end complex. Whether DTT's inhibitory action is aimed directly at the enzyme-substrate complex or at sites of the free enzyme protein remains to be investigated. To elucidate the exact role of DTT in 5'-deiodination, be it

stimulatory or inhibitory, it could be fruitful to consider biochemical actions of DTT other than that as a strictly cysteine-reducing agent. Also, amino acid residues other than cysteine, for example methionine (Vogt 1995), are redox-sensitive and can potentially confer a specific DTT sensitivity to a deiodinase protein.

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Chapter 7

Peripheral regulation of thyroid physiology upon nutritional challenges in common carp (*Cyprinus carpio* L.).

The involvement of factors other than food availability.

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Submitted to *Aquaculture*

Abstract

Classically thyroid hormones are regarded as major determinants of metabolic rate in vertebrates. Also in teleost fish, thyroid hormones are implicated in metabolic processes, and this is exemplified by their responsiveness upon fasting when the peripheral thyroid system is down-regulated. In this study we investigated the effects of nutritional challenges, *viz*. 6 weeks fasting and feeding ad libitum, on the peripheral thyroid system of common carp (*Cyprinus carpio*). We have measured free thyroid hormone plasma levels and renal and hepatic deiodinase activity and expression. Upon 6 weeks of fasting, plasma free T3 (fT3) levels were reduced by 57% whereas fT4 levels remained unchanged. This reduction in plasma fT3 was accompanied by a 72–85% and 67% decrease in renal deiodinase activity and D2 expression, respectively, whereas hepatic deiodinase activity and expression remained relatively unchanged. Despite a 4 to 2-times increase in food consumption upon feeding ad libitum, the peripheral thyroid system, as well as the specific growth rate (SGR), remained unchanged. This suggests that upon nutritional challenge the peripheral thyroid system relates to growth rather than food intake. Time course analysis of peripheral thyroid parameters during the fasting period, revealed that a factor other than nutritional status is involved in the regulation of the thyroid status. We postulate that the decrease in stocking density during the fasting period, likely resulting in changed group dynamics, may be responsible for the observed confounding effect. We conclude that changes in plasma thyroid hormone levels upon fasting in common carp are regulated through the action of renal type 2 deiodinase. Furthermore, changes in the peripheral thyroid system upon nutritional challenges are likely to be involved in long-term metabolic processes such as growth and may be affected by other factors, for instance changes in group dynamics due to varying stocking densities.

Introduction

The actions of thyroid hormones are pleiotrope and include the regulation of reproduction, hydromineral balance, growth, ontogenetic development and metabolic processes. Thyroid hormones are regarded as major determinants of energy homeostasis in endothermic vertebrates, which is perhaps best exemplified by the increased and decreased metabolic rate in, respectively, hyperthyroid (Møller, et al. 1996) and hypothyroid patients (Wolf, et al. 1996). Also in ectothermic vertebrates thyroid hormones have been implicated in the regulation of metabolic rate. In teleost fish, thyroid hormones increase oxygen consumption, exert gluconeogenic effects, increase lipolytic enzyme activity and have anabolic effects on protein metabolism (Ballantyne, et al. 1992; Lynshiang and Gupta 2000; Pandey and Munshi 1976; Peter and Oommen 1989; Ruhland 1969; Scott-Thomas, et al. 1992; Shameena, et al. 2000; Sheridan 1986), although negative and contradicting results have also been reported (see review by Plisetskaya, et al. 1983).

The involvement of thyroid hormones in teleost metabolism is further corroborated by the effects of nutritional challenges on thyroid physiology. For example, in several teleost species plasma levels of T4 and T3 decrease upon fasting (Cerda-Reverter, et al. 1996; De Pedro, et al. 2003; De Pedro, et al. 1995; Finnson and Eales 1999; Raine, et al. 2005; Van der Geyten, et al. 1998). In line with these results is our observation on the hyperfagous behaviour of common carp (*Cyprinus carpio* L.) that were treated with T4 (Geven, et al. 2006). Thyroid hormones appear to influence energy homeostasis and metabolism in fish *via* both energy expenditure and food (energy) intake.

The extra-thyroidal conversion of T4 into the bio-active iodothyronine T3 via outer ring deiodination (ORD) is crucial for proper thyroid hormone function. In vertebrates ORD is catalysed by deiodinase type 1 (D1) and type 2 (D2), which are therefore important determinants for circulating plasma T3 levels (Bianco, et al. 2002; Gereben, et al. 2008). In teleost fish peripheral deiodinase activity is affected by starvation; in rainbow trout (*Oncorhynchus mykiss*) the hepatic conversion of T4 to T3 had decreased (Finnson and Eales 1999; Leatherland and Farbridge 1992; Shields and Eales 1986) and also in brook trout (*Salvelinus fontinalis*) indirect evidence suggests a decrease in the conversion of T4 to T3 upon food deprivation (Higgs and Eales 1977). In fasted

Nile tilapia (*Oreochromis niloticus*) hepatic D2 activity is down regulated (Van der Geyten, et al. 1998), suggesting that in fish hepatic D2 activity is responsible for the changes in plasma T3 levels during fasting.

In this study we have investigated the effect of three feeding regimens, *viz*. fasting, feeding at a restricted ration of 2% of the estimated body weight, and feeding ad libitum, on the peripheral regulation of thyroid physiology in common carp (*Cyprinus carpio*). These feeding regimens have been shown to evoke marked physiological responses in common carp (Huising, et al. 2006). Experimental read-outs included free T3 and free T4 plasma concentrations, and the mRNA expression and ORD activity of hepatic and renal D1 and D2. In addition to an endpoint analysis, we also investigated the time course of these read-outs during the fasting period.

Materials and methods

Animals

Common carp (*Cyprinus carpio* L.), of the all-male E4×R3R8 isogenic strain (Bongers, et al. 1998) were obtained from the Department of Fish Culture and Fisheries of Wageningen University (The Netherlands). Fish were kept in 140-L tanks with circulating aerated city of Nijmegen tap water at 23°C, at a photoperiod of 16 hours light and 8 hours darkness, and were fed dry food pellets (LDX Filia slow sinking; Trouw Nutrition International, Putten, The Netherlands) once daily, except for the animals fed ad libitum (see below). Animals were weighed weekly and the food ration was adjusted accordingly. Plasma was collected from fishes that were terminally anesthetised with 0.1% (v/v) 2-phenoxyethanol (Sigma Chemical Co., St. Louis, MO, USA). Immediately afterwards fish were killed by spinal transection, and selected tissues were dissected. Animal handling followed approved university guidelines.

Feeding regimen

The effects of long-term fasting on carp thyroid physiology were studied in food-deprived animals (fasted group, n=66) and were compared to animals fed a daily ration of 2% of their estimated body weight (normally fed group, n=58). Both groups were kept in two tanks with separate filtering systems and were acclimated to a daily food ration of 2% of their body weight, which was delivered once daily at 09:00 h. One hour after feeding 8 animals were sampled which constituted the time-point zero control group. From this time-point onwards, the animals from the fasted group received no food for 6 weeks, whereas the normally fed group was maintained at the initial feeding regimen.

The effects of ad libitum feeding were studied in animals fed to satiation for 6 weeks (ad libitum group, n=16) and were compared to fish fed a daily ration of 2% of their estimated body weight (normally fed group, n=16). The feeding-to-satiation regimen consisted of an initial ration of 2% of the estimated body weight delivered at 09:00 h, followed by the continuous distribution of food pellets with an automated feeder throughout the 16-h light period. The daily amount of food consumed during the first week of this regimen was 7.5% of the estimated body weight, and decreased to 5.0% in week 2 and 3.7% in week 6. After 6 weeks 8 animals from each group were sampled 1 h after the delivery of the first ration.

RNA isolation & real-time quantitative PCR

The kidney and liver were removed, snap frozen on dry ice and stored at -80°C until further analysis. Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). After removal of residual DNA with a DNase treatment (Invitrogen), 1 μg of RNA was reverse transcribed to cDNA with Superscript II reverse transcriptase (Invitrogen) and stored at -20°C. A control containing no reverse transcriptase was included for each sample.

The relative mRNA expressions of D1 and D2 in liver and kidney were determined by real-time quantitative PCR (RQ-PCR). In short, 5 μl of 25 times diluted cDNA was used in a reaction mixture consisting of SYBR Green Master Mix (PE Applied Biosystems Benelux, Nieuwerkerk aan den IJssel, The

Netherlands) and a primer set as shown in Table 1. Melting curves confirmed the identity and specificity of the PCR reactions. Dissociation plots were analyzed and cycle threshold (Ct) values were determined. The expression of the genes of interest was calculated relative to β-actin and 40S ribosomal protein S11 mRNA expression. Both control genes yielded similar results and therefore all results in this study are expressed relative to 40S mRNA expression.

Plasma parameters

Blood was sampled by puncture of the caudal vessels with a syringe fitted with a 23-G needle rinsed with a solution of 1 mM ethylenediamine tetraacetic acid (EDTA, Sigma Chemical Co.). Plasma was obtained after centrifugation at 4°C (4000 *g*, 15 min) and stored at -20°C until further analysis. Plasma free-T4 (fT4) and free-T3 (fT3) levels were determined with a Delfia® solid-phase time-resolved fluoroimmunoassay (TR-FIA) from PerkinElmer (Waltham, MA, USA) and measured in a Wallac Victor^2 multilabel counter 1420 (PerkinElmer). Calibration curves were satisfactorily described by a log-log and a log-logit transformation of the T3 and T4 standards, respectively, and fluorescence counts. Serial dilutions of carp plasma with charcoal-stripped carp plasma produced displacement curves parallel to those prepared from dilutions of the T3 and T4 calibrators supplied with the Delfia® kit. The intra- and inter-assay variation for the fT3 and fT4 TR-FIA are 4.9% and 4.6%, and 3.3% and 4.0%, respectively, as reported by the manufacturer.

Outer ring deiodinase assay

Liver and kidney were homogenized in 2 ml phosphate buffer (100 mM Na-phosphate at pH 7.2, 2 mM EDTA) using a Potter-Elvehjem device. Outer ring deiodinase (ORD) activity in these homogenates was determined as described previously, with substrate concentrations of 10 μM (Klaren, et al. 2005). In preliminary experiments we established the requirements of the rT3-, T4- and T3-ORD reactions for dithiothreitol (DTT). Dithiothreitol was found to be a

Table 1. Primers sequences with corresponding GenBank accession numbers.

Gene product	Accession nr.	Primer	Sequence 5′ → 3′
D1	-	qD1-fw	TGCGATCGTGAATGTAACCC
		qD1-rv	GAGTCATCGTCGACTTCTCT
D2	-	qD2-fw	ATGGAACAGCTTTCTGCTGG
		qD2-rv	TAGACAAGCAGGAAGTCTGC
β-actin	M24113	qACT-fw	CAACAGGGAAAAGATGACACAGATC
		qACT-rv	GGGACAGCACAGCCTGGAT
40S	AB012087	q40S-fw	CCGTGGGTGACATCGTTACA
		q40S-rv	TCAGGACATTGAACCTCACTGTCT

potent inhibitor of ORD activities in carp kidney homogenates. Preparations from liver, however, required an optimum concentration of 1 mM DTT (results not shown). Consequently, DDT was omitted from the kidney homogenate incubations, and was added to a concentration of 1 mM in the assay media containing liver homogenates.

Calculations and statistical analysis

Specific growth rates (SGR, d^{-1}) were calculated as:

$$SGR = \frac{100 \times \ln(W_2/W_1)}{t_2 - t_1}$$

Where W_2 and W_1 are the body weights (g) measured on time points t_2 and t_1 (days), respectively. Other data are presented as mean values ± standard deviation. Differences between two groups were assessed with Student's unpaired *t*-test, or Welch's *t*-test to analyse averages with unequal variances. Multiple groups were compared by one-way ANOVA and Tukey's post-hoc test. Statistical significance was accepted at $P < 0.05$ (two-tailed), probabilities

are indicated by asterisks (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$) or by different letters ($P < 0.05$).

Results

Plasma free iodothyronine levels

After six weeks of fasting, plasma fT3 levels had significantly decreased by 57% ($P < 0.05$) compared to plasma levels of normally fed fish (Fig. 1A). Plasma levels of fT4, however, remained unaffected (Fig. 1A) and were comparable to previously reported values for plasma fT4 in common carp (Geven, et al. 2006). Feeding to satiation for 6 weeks did not significantly change plasma fT4 and fT3 levels when compared to normally fed fish (Fig. 1B). The plasma levels of fT3 and fT4 of the control groups in both experiments did not differ significantly.

Deiodinase expression and activity after fasting

After six weeks of fasting, the expression of D2 mRNA in kidney was down-regulated by 67% ($P < 0.001$) (Fig. 2A). This correlated positively with the renal ORD activities towards rT3, T4 and T3 which were reduced by 72 to 85% ($P <$ 0.05-0.01) (Fig. 2B). In liver the effect of fasting was less pronounced. The expression of D1 mRNA was down regulated by 26% ($P < 0.05$) (Fig. 2C), but this was not reflected in the 1.4-fold increased hepatic rT3 ORD activity (Fig. 2D).

Deiodinase expression and activity after feeding ad libitum

Feeding *ad libitum* for 6 weeks resulted in few and inconsistent changes in renal and hepatic deiodinase expression and ORD activity. Renal D1 expression decreased by 23% ($P < 0.05$) (Fig. 3A), while renal ORD activities remained unchanged (Fig. 3B). In liver, the expression of deiodinases remained unaffected (Fig. 3C), although hepatic rT3- and T4-ORD activities were significantly reduced by 48 and 42%, respectively ($P < 0.01$) (Fig. 3D).

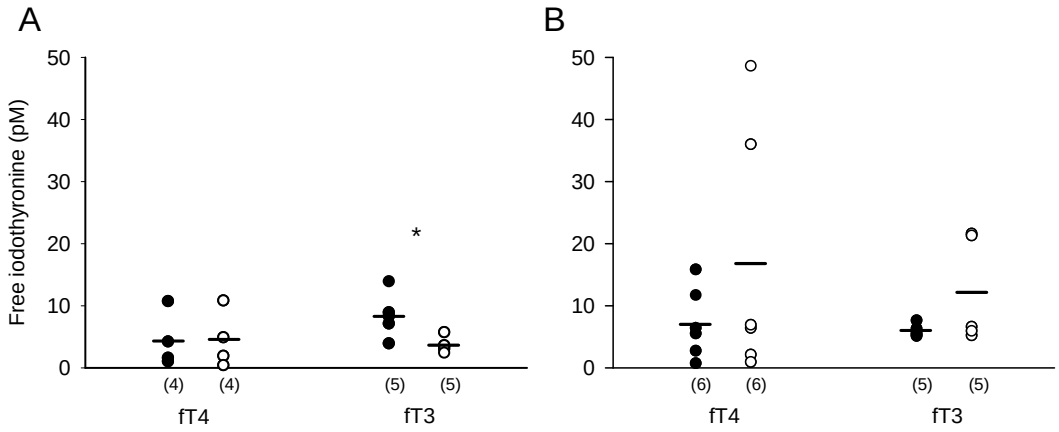

Figure 1. Plasma concentrations of free T4 (fT4) and free T3 (fT3) in 6-weeks fasted carp (panel A, open symbols) and in carp fed for 6 weeks ad libitum (panel B, open symbols) compared to normally fed carp (control, closed symbols). Sample size is in parentheses, horizontal bars represent mean values.

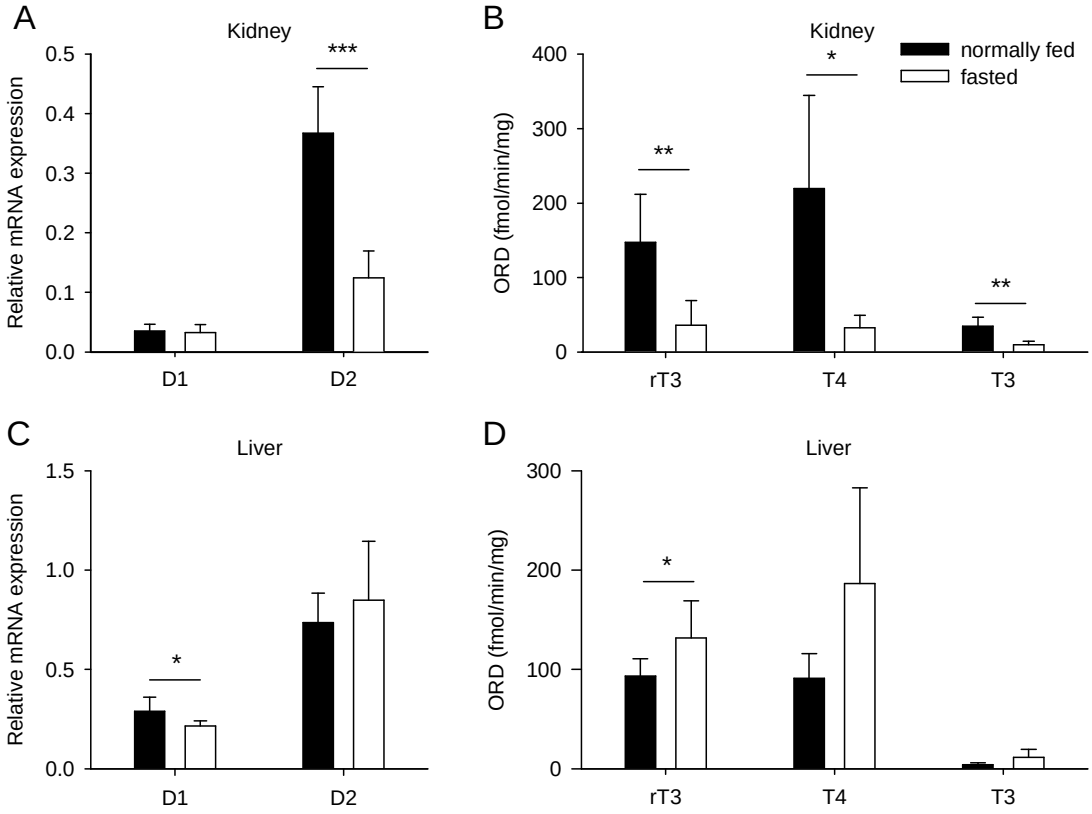

Figure 2. The relative mRNA expression of D1 and D2 (A, C, n=8) and ORD activities towards rT3, T4 and T3 (B, D) (n=5-6) in kidney (A, B) and liver homogenates (C, D) of carp fasted for 6 weeks (open bar) compared to normally fed carp (control, closed bars).

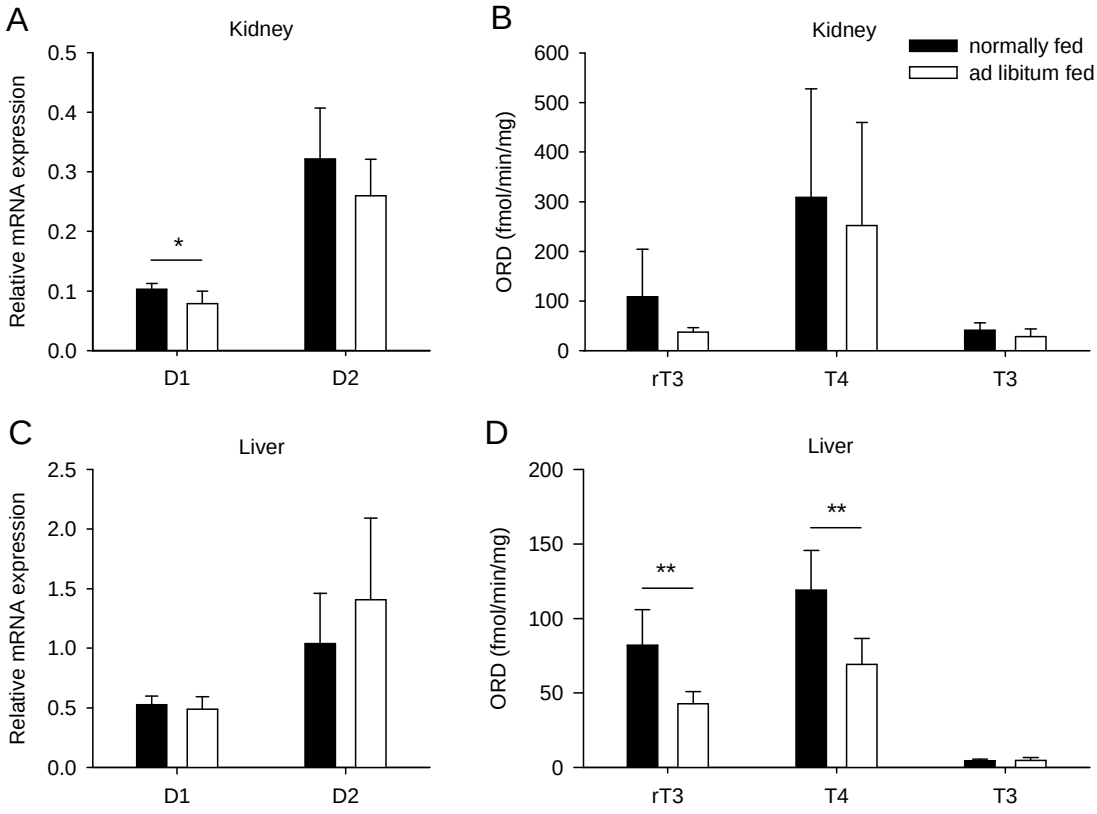

Figure 3. The relative mRNA expression of D1 and D2 (A, C, n=8) and the ORD activity towards rT3, T4 and T3 (B, D) (n=5-6) in kidney (A, B) and liver homogenates (C, D) of carp fed ad libitum for 6 weeks (open bar) compared to normally fed carp (control, closed bars).

Time courses of the specific growth rates, plasma thyroid hormone concentration and deiodinase expression and activity

To investigate the time-course of the changes in the peripheral thyroid system we also sampled and measured at intermediate time-points during the fasting period. The plasma fT4 concentration did not differ significantly between fasted and normally fed fish during the 6-weeks fasting period (Fig. 4A), but that of fT3 did. During fasting, plasma fT3 concentrations in the normally fed control group increased, whereas plasma fT3 remained relatively unchanged in the experimental, fasted group. This resulted in a 51% to 77% ($P < 0.05$) net

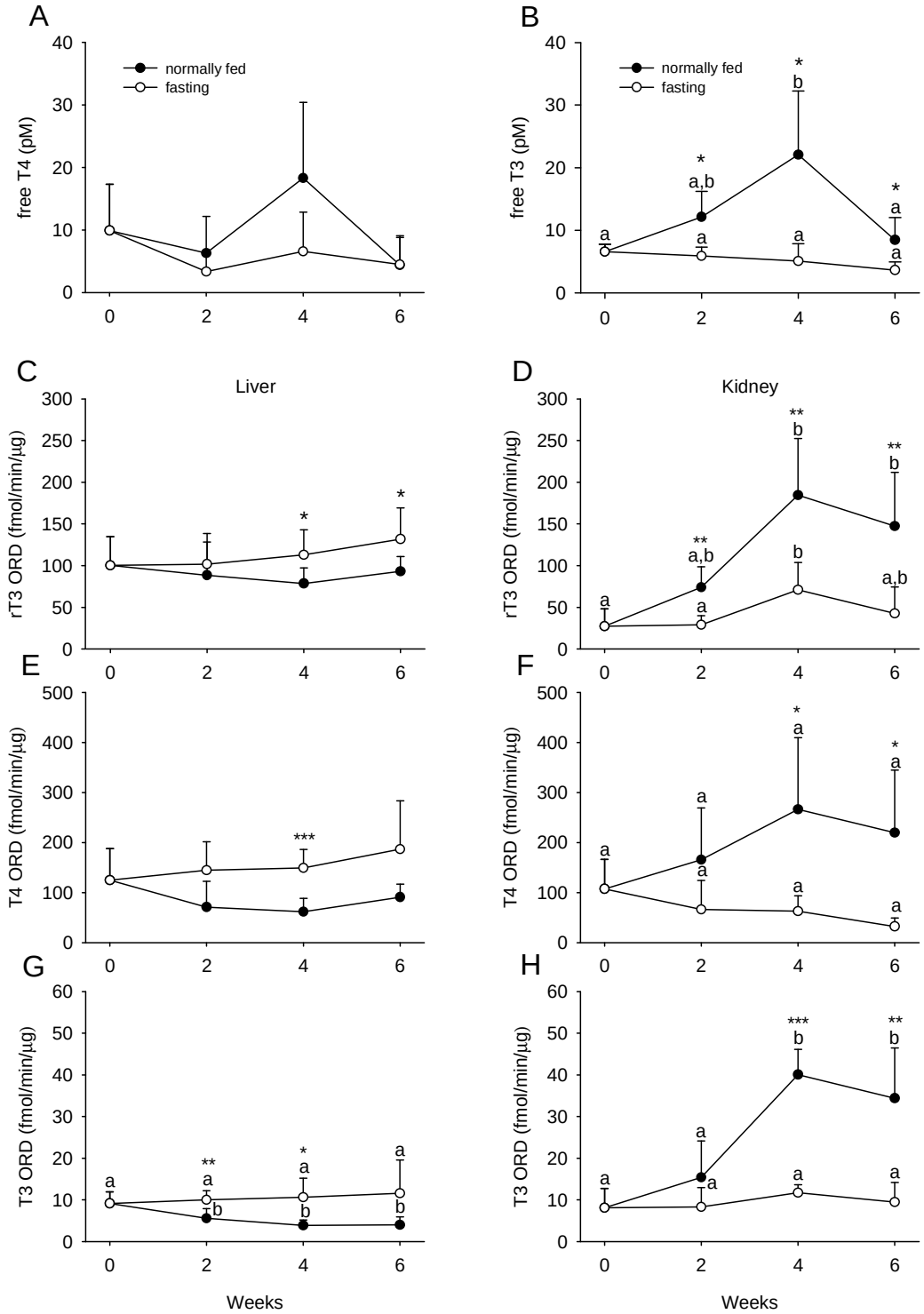

Figure 4. Plasma concentrations of fT4 (A) (n=4-6) and fT3 (B) (n=4-6) in fasted carp during a 6 weeks period (open symbols) compared to normally fed carp (closed symbols). The profile of ORD-activity in liver (C, E, G) and kidney (D, F, H) homogenates for rT3 (C, D), T4 (E, F) and T3 (G, H) of carp fasted for 6 weeks (open symbols) (n=3-6) compared to normally fed carp (closed symbols) (n=3-6).

Table 2. Specific growth rates (SGR, d^{-1}) of fasted carp and carp fed ad libitum compared to normally fed carp.

Time period (weeks)	Fasting (0%)	Normal feeding (2%)	Normal feeding (2%)	Ad lib. feeding (7.5 – 3.7%)
0 – 2	0.1	2.0	1.8	5.5
2 – 4	– 0.4	2.1	1.7	2.2
4 – 6	– 1.0	1.3	1.8	2.3

decrease of fT3 in the fasted group compared to the normally fed controls at 2, 4 and 6 weeks (Fig. 4B). Similarly, renal ORD activity in the fasted animals remained constant during the experimental period, but increased in the normally fed, control group (Fig. 4D, F, H). Hepatic ORD activities were relatively stable during the fasting period, and were generally higher than those in the normally fed control animals (Fig. 4C, E, G).

The specific growth rate (SGR) of fasted carp approached a value of zero within 2 weeks, the negative SGRs observed following this first 2-week fasting period indicate weight loss (Table 2). The SGR of normally fed carp in both the fasting and ad libitum feeding experiment did not significantly differ and varied between 2.1 and 1.3 d^{-1}. Ad libitum feeding produced increased SGRs, which initially reached 5.5 d^{-1} in the first two weeks, after which the SGR declined to values approaching that of normally fed carp (Table 2), while food intake was still 2 times higher in the ad libitum fed fish compared to normally fed fish.

Discussion

Fasting common carp for 6 weeks resulted in a decreased concentration of plasma fT3 compared to control fish, while plasma levels of fT4 remained unaffected. The decrease in plasma fT3 was accompanied by marked decreases in renal ORD activity and D2 mRNA expression. These results show that the kidney, through the action of D2, is the key tissue that regulates circulating fT3 levels upon a decreased nutritional status in common carp.

Our results corroborate previous work by Van der Geyten, et al. (1998) who showed that in teleost fish D2 is pivotal in the control of plasma T3 levels during fasting (Van der Geyten, et al. 1998) and not D1 as in mammals (Aceves, et al. 2003; O'Mara, et al. 1993; Santini and Chopra 1992). In common carp, food ration responsive deiodinases reside in the kidney, whereas in other species (rainbow trout, *Oncorhynchus mykiss*, and Nile tilapia, *Oreochromis niloticus*) they are located in the liver (Finnson and Eales 1999; Leatherland and Farbridge 1992; Van der Geyten, et al. 1998).

In common carp the kidney not only harbours nephronic vascular and tubular structures, it also includes the functional endocrine thyroid gland (Geven, et al. 2007). Consequently the ORD activity measured in the kidney can either be of nephronic or thyroidal origin. ORD activity has indeed been observed in mammalian and teleostean thyroid tissue (Chanoine, et al. 1993; Plohman, et al. 2002), which in rodents is down-regulated upon fasting (Lisboa, et al. 2007; Wu 1990). Furthermore, in food-deprived rainbow trout, 75 - 81% of plasma T3 is secreted by the thyroid gland, while the remaining is derived by extra-thyroidal deiodinase activity (Sefkow, et al. 1996). How this percentage relates to that in normally fed rainbow trout remains unknown, but it is generally assumed that the thyroid gland secretes minimal amounts of T3 in teleosts (Eales and Brown 1993). It may be that in fish the role of extra-thyroidal deiodination in determining plasma thyroid hormone levels becomes less prominent during fasting and is taken over by thyroidal deiodinases. It can tentatively be suggested that this mechanism allows for the quick recycling of iodide within the thyroid gland, and bypasses the energy-dependent sodium-iodide symporter in the basolateral membrane of the thyrocyte.

Whereas the role of kidney ORD in the regulation of thyroid hormone availability in common carp during fasting is evident, the role of the liver ORD is less apparent. Hepatic ORD is clearly not involved in the regulation of plasma T3, since upon fasting only minor changes are observed. Upon feeding ad libitum, hepatic ORD activity is increased while plasma thyroid hormones levels remained unaffected. Since the liver fulfils a major role in the regulation of the intermediary metabolism, these changes in hepatic ORD activity most likely reflect changes in intracellular T3 to facilitate adaptive responses in hepatic processes involved in carbohydrate, protein or lipid metabolism.

Whereas peripheral thyroid parameters, *viz.* plasma fT3 and renal ORD activity, are responsive to food deprivation, they do not change during an ad libitum feeding regimen. Food intake alone does not reflect energy input per se, and processes such as nutrient absorption, conversion and incorporation have to be taken into account. Growth, which is the net difference of energy input and energy expenditure, is therefore a better reflection of the animal's nutritional status than food intake. Despite the 2 to 4 times higher food intake in the ad libitum fed carp, the SGR, after an initial rise, after 5 to 6 weeks approached the SGR of the normally fed carp. It appears that the peripheral systemic thyroid status is not so much correlated to the amount of food consumed but rather to the SGR.

A relation between thyroid status and growth has been established in several teleost species. In rainbow trout and Arctic charr (*Salvelinus alpinus*) plasma levels of T3 correlated with the SGR and not with the amount of food intake, while plasma levels of T4 did not correlate with either (Eales and Shostak 1985; Gomez, et al. 1997). Also in Nile tilapia (*Oreochromis niloticus*) and lake sturgeon (*Acipenser fulvescens*) SGR correlated with plasma T3 and not with plasma T4 (Plohman, et al. 2002; Toguyeni, et al. 1996). As in common carp, in Arctic charr overfeeding (3-4% of estimated body weight) did not result in an increased SGR or plasma T3 levels (Eales and Shostak 1985). Furthermore, it has been shown that in lake sturgeon SGR is negatively correlated with T3 deiodinase activity in the intestine and liver (Plohman, et al. 2002), showing that, as in common carp, the changes in plasma T3 at different growth rates are due to changes in the peripheral deiodinase activity.

The end-point analysis after 6 weeks of nutritional challenge clearly shows that plasma fT3 levels and renal ORD activity are correlated to growth rather than food intake. The measurement of these parameters at intermediate time points in fasted carp reveals that a factor other than nutritional status, interferes with the peripheral thyroid status. Plasma fT3 and renal ORD activity did not remain unchanged in the control group in the fasting experiment despite the unchanged 2% feeding regimen and a constant SGR. Both parameters increased in normally fed carp, reaching maximum values after 4 weeks.

A possible confounding factor is the gradual decline in the stocking density, which is inherent to the experimental design of this study in which repeated sampling took place. Numerous aqua-cultural studies have shown that increased stocking densities adversely affect several physiological processes in fish, including thyroid physiology (Portz, et al. 2006). Rainbow trout reared at relative high stocking densities (150 kg/m^3 compared to 60 kg/m^3) displayed significant decreases in plasma T3, liver T3 content and hepatic T4-ORD activity (Leatherland 1993). At even higher densities (277 kg/m^3 compared to 134 kg/m^3) plasma T4 levels were decreased as well (Leatherland and Cho 1985). Since the densities in this study (33 kg/m^3 at week 0 and 12 kg/m^3 at week 6) are significantly lower than in aqua-cultural settings, density-associated factors such as water quality or competition for food appear not to be implicated, species differences between carp and salmonids notwithstanding. More likely, reduced agonistic social stress or other changes in group dynamics are responsible for the observed changes in thyroid physiology in this study. Although plasma fT3 and renal ORD activity in the fasted carp remain relatively unaltered, in fact these parameters are affected by two counteracting factors, *viz.* a decrease in density and food deprivation. Clearly, the regulation of the thyroid system in fish during a nutritional challenge is dependent on a multitude of factors, *e.g.* food intake, growth and, possibly, group dynamics.

Addressing the physiological relevance, it appears that the reduced peripheral thyroid system in fasted fish is not involved in the short-term redistribution of energy, as the changes are slow and gradual. Changes in plasma fT3 and renal ORD reach maximum difference only after 4 weeks, while changes in the intermediary metabolism are immediate, *e.g.* the transition from a carbohydrate to a lipid metabolism is already established after 24 hours of fasting in common carp (Huising, et al. 2006). Although thyroid parameters were not measured in the first two weeks of the fasting period, it appears that upon fasting the peripheral thyroid system is involved in long-term metabolic processes, e.g. the incorporation of energy in somatic growth, rather than immediate metabolic processes.

In summary, the changes in plasma thyroid hormone levels observed in the nutritionally challenged common carp reflect changes in growth and not changes in food intake or primary metabolic processes. These changes in

plasma TH levels are regulated through the action of renal type 2 deiodinase and may be affected by other factors then growth rate, for instance changes in group dynamics due to varying stocking densities.

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Chapter 8

Summary and general discussion

In this thesis several components of the thyroid system in common carp are described, ranging from the central regulation of thyroid gland activity to the biochemical characterisation of peripheral deiodinases. The main findings and interpretation will here be discussed, and include the following;

1. In juvenile and adult common carp the functional thyroid gland is located in the renal tissues. Thyroid follicles in the subpharyngeal region do not accumulate iodide or release T4 upon treatment with TSH, but they do exhibit T4-immunoreactivity, which suggests functionality of these at earlier life stages. Indeed, in larval common carp the thyroid gland is first developed in the subpharyngeal region, where, prior to follicle formation, thyroid hormones are synthesised intracellularly.
2. The HPT-axis in common carp interacts with the HPI-axis and the melanocortin system, centrally as well as peripherally.
   - Thyroid hormones modulate the hypothalamic CRH-system, as T4 stimulates the expression of CRH-BP and CRH mRNA *in vitro*. Conversely, CRH does not regulate HPT-axis activity, as it does not affect TSH expression. Peripherally, ACTH and cortisol stimulate the release of thyroid hormones *in vitro* from head kidney and kidney. Vice versa, thyroid hormones do not stimulate the release of cortisol from head kidney.
   - Centrally, thyroid hormones stimulate the expression of TRH, which, subsequently, results in the increased expression of pituitary POMC and elevated levels of plasma α-MSH. Peripherally, α-MSH stimulates the release of thyroid hormones from head kidney and kidney.
3. Deiodinase activity in common carp is responsive to prolonged fasting. Hepatic and renal ORD activities display profound biochemical and kinetic differences and were assayed under validated tissue-specific conditions. Upon fasting, renal ORD activity and D2 expression are down-regulated, which correlated with decreased plasma levels of free T3. Hepatic ORD

activity and deiodinase expression do not correlate with the changes in plasma thyroid hormones.

The following discussion will focus on the findings related to the phenomenon of thyroid heterotopia, the interaction of the HPI- and the HPT-axis and their possible functional implications and the integrative neuroendocrine control of thyroid gland activity.

Thyroid heterotopia

The term "heterotopic" or "ectopic" is used to describe thyroid follicles located outside their typical location which, in vertebrate species, is generally assumed to be the ventral pharyngeal region. The term also suggests that this distribution is an anomaly, and that their physiological relevance is secondary or at best supplementary to the total thyroid output. The "atypical" location of a functional thyroid gland in the renal tissues and the lack of active thyroid follicles in the "typical" subpharyngeal region in carp (**chapter 2**), show that, at least in this species, the term heterotopic is a misnomer for renal thyroid follicles.

Since during early development thyroid follicles are exclusively observed in the subpharyngeal region (**chapter 3**), a translocation of thyroid tissue from the subpharyngeal region towards the renal tissues can be inferred. Two important questions remain to be elucidated, namely how and why thyroid follicles emerge in the renal tissues.

Mechanisms for thyroid heterotopia

Baker (1958a, b), who was the first to investigate thyroid heterotopia in a teleost fish (platyfish, *Xiphophorus maculates*), suggested three mechanisms for the emergence of thyroid follicles in the renal tissues.

I. During early development, non-differentiated thyroid cells end up in renal tissue which differentiate into thyroid follicles.

II. Dissociated thyrocytes are transported from the subpharyngeal region to the renal tissue via the blood stream.
III. Thyroid cells or follicles migrate via the walls of blood vessels to their ultimate destination.

Although neither suggestion can yet be excluded, the third mechanism appears most plausible, because of the strong association *in situ* of the subpharyngeal thyroid follicles with the ventral aorta and branchial arteries in fish (Gudernatsch 1911). Moreover, in a zebrafish mutant with an impaired pharyngeal vascular architecture, thyroid follicles did not develop along the anterior-posterior axis of the ventral aorta, but extended more laterally (Alt, et al. 2006). Grafting of ectopic vascular cells in zebrafish during late blastula stage affected thyroid localisation: the thyroid follicles projected towards nearby located grafted vascular cells (Alt, et al. 2006). It appears that the pharyngeal vascular system provides chemotactic cues that guide the organisation of thyroid tissue along the ventral aorta. A specific expression pattern of these signals along the inferior jugular vein, sinus venosus, cuverian duct and the cardinal veins may facilitate the specific migration of thyroid tissue towards the renal tissues.

Thyroid heterotopia; compensatory to hypothyroidism

More fundamentally and physiologically relevant than the question how thyroid follicles emerge in extra-pharyngeal sites, is the question why thyroid follicles become heterotopic. Phylogenetic analysis does not provide indications for their occurrence, as renal thyroid heterotopia is widely distributed and not confined to a specific clade. Also, thyroid heterotopia is found in freshwater and seawater fish alike (Table 1, Figure 1). Remarkable is the high incidence of renal heterotopic thyroid follicles in the order of the Cyprinidae, suggesting it may be induced by a shared cyprinid trait, although not all cyprinid species, e.g. zebrafish (personal unpublished observation), display thyroid heterotopia.

Baker suggested that thyroid heterotopia is a compensatory mechanism to cope with iodine deficiency, as the administration of surplus iodine to the water

resulted in the developmental arrest of heterotopic thyroid follicles in platyfish (Baker 1958b). Although this deduction appears plausible, several critical notes have to be made. First of all, the platyfish used by Baker belonged to a specific strain (*BH*-strain) that is highly susceptible for thyroid tumour development. Therefore we have to consider pathological mechanisms, e.g. metastasis, to be involved in the platyfish thyroid heterotopia. Secondly, a 1000-fold higher concentration (8 μM) of iodine, as compared to the control situation (8 nM), was applied. Since iodine concentrations in the micromolar range are not typical for natural aquatic habitats (freshwater; 0.5-200 nM, seawater 350 nM^{-1} μM) (Schwehr and Santschi 2003) one could argue whether the emergence of heterotopic thyroid follicles is truly a compensatory mechanism for iodine deficiency within a physiological context. The availability of iodine in a water body is virtually infinite, and there is thus a continuous and most probably sufficient supply of iodine. Moreover, fish are highly efficient in the uptake of iodine from water. Both active transport, mediated by the sodium-iodide symporter, and passive transport of iodine, possibly via the gill epithelium, have been reported in fish (Moren, et al. 2008, **chapter 2**). It therefore remains questionable whether fish can actually experience "environmental" hypothyroidism caused by low concentrations of iodine in the ambient water.

Not only iodine deficiency may lead to hypothyroidism, also an impaired biosynthesis of thyroid hormone may be an underlying principle that explains thyroid heterotopia. Indeed, from functional and developmental studies (**chapter 2 & 3**), we infer that the subpharyngeal thyroid follicles in common carp have turned dormant during development, which may have initiated the development of heterotopic thyroid tissue. However, this does not provide a general explanation for thyroid heterotopia, as in all other fish species investigated with functional heterotopic thyroid follicles, the subpharyngeal thyroid was active (Bhattacharya, et al. 1976a; Chavin and Bouwman 1965; Frisén and Frisén 1967; Peter 1970; Srivastava and Sathyanesan 1971b).

For these fish, where the subpharyngeal thyroid is functional, it remains the question whether hypothyroidism indeed causes thyroid heterotopia. Why does thyroid tissue not expand within the subpharyngeal region but instead translocates

to other tissues? Expansion of thyroid tissue in the subpharyngeal region is not physically restrained by e.g bone structures, and, moreover, the non-encapsulated nature of the teleost thyroid gland should allow for easy proliferation. Indeed, there are several reports on a goitrous thyroid gland within the subpharyngeal region in fish that have been subjected to xenobiotic disruptors (Moccia, et al. 1977, 1981). Clearly, the translocation of thyroid tissue to extra-pharyngeal locations may serve other functions than merely the production of extra thyroid hormones.

Thyroid heterotopia; integration of endocrine signals

Another relevant question is why heterotopic thyroid follicles are preferentially located in the renal tissues. Whereas Baker tried to explain thyroid heterotopia from a valid thyroid perspective, e.g. an arrested thyroid hormone synthesis due to iodine deficiency, we follow an integrative endocrinological approach from the renal perspective. This renal context of heterotopic thyroid follicles is the presence of other endocrine cell types and structures with which thyrocytes can interact via local, paracrine mechanisms. These cell types and structures observed in renal tissues include nephronic structures in the trunk kidney which are involved in the regulation of the hydromineral balance. In (head) kidney, hematopoietic tissue is present from which progenitors of the immune system derive. Furthermore, the head kidney contains interrenal cells and chromaffin cells which produce the stress hormones cortisol and catecholamines. We therefore postulate that the "raison d'être" for heterotopic thyroid follicles is the interrelationship of the HPT-axis with either the osmoregulatory system, the immune system, the HPI-axis or any combination thereof. The latter was investigated in this thesis and will be discussed in the following paragraph.

Table 1. Fish species described in the literature with heterotopic thyroid follicles in renal tissues.

Order	Family	Species	References
Cyprinidae			
	Cypriniformes	Common carp (*Cyprinus carpio*)	(Chavin 1966; Lysak 1964; Qureshi and Sultan 1976; Sugiyama and Sato 1960)
		Goldfish (*Carassius auratus*)	(Chavin 1956a, b; Chavin and Bouwman 1965; Peter 1970; Qureshi, et al. 1978)
		Crucian carp (*Carassius carassius*)	(Frisén and Frisén 1967)
		Silver carp (*Hypophthalmichthys molitrix*)	(Kruger 1991)
		Ide (*Leuciscus idus*)	(Kruger 1991)
		Pool barb (*Puntius sophore*)	(Agrawala and Dixit 1979; Sathyanesan 1963; Srivastava and Sathyanesan 1971b)
		Iraq blind barb (*Typhlogarra widdowsoni*)	(Olivereau 1960)
		Mahseer (*Tor tor*)	(Qureshi and Sultan 1976)
		Mrigal (*Cirrhinus cirrhosus*)	(Joshi and Sathyanesan 1976)
		Garra (*Garra lamta*)	(Pandey 1964; Qureshi, et al. 1978)
		Catla (*Catla catla*)	(Ahuja and Chandy 1962)
		Rosy barb (*Puntius conchonius*)	(Sathyanesan and Prasad 1962)
		Rohu (*Labeo rohita*)	(Kulkarni and Sathyanesan 1978)
		Cherry barb (*Puntius titteya*)	(Baker 1959)
		Ticto barb (*Puntius ticto*)	(Bose and Ahmad 1977)
Siluriformes			
	Clarridae	Walking catfish (*Clarias batrachus*)	(Bhattacharya, et al. 1976b; Sharma and Kumar 1982)
	Bagridae	Striped dwarf catfish (*Mystus vittatus*)	(Bose and Ahmad 1978; Gurumani 1971)
	Heteropneustidae	Stinging catfish (*Heteropneustes fossilis*)	(Qureshi 1975; Qureshi and Qureshi 1974)
Perciformes			
	Channidae	Spotted snakehead (*Channa punctata*)	(Bhattacharya, et al. 1976a)
	Gobiidae	Tank goby (*Glossogobius giuris*)	(Qureshi, et al. 1978)
	Polynemidae	Sixfinger threadfin (*Polydactylus sexfilis*)	(Qureshi, et al. 1978)
Cyprinodontiformes			
	Poeciliidae	Southern platyfish (*Xiphophorus maculates*)	(Baker 1958a, b; Baker, et al. 1955)
		Montezuma swordtail (*Xiphophorus montezuma*)	(Baker 1959)
Synbrachiformes			
	Synbranchidae	Cuchia (*Monopterus cuchia*)	(Srivastava and Sathyanesan 1967, 1971a)
Clupeiformes			
	Engraulidae	Gangetic hairfin anchovy (*Setipinna phasa*)	(Sathyanesan and Chary 1962)

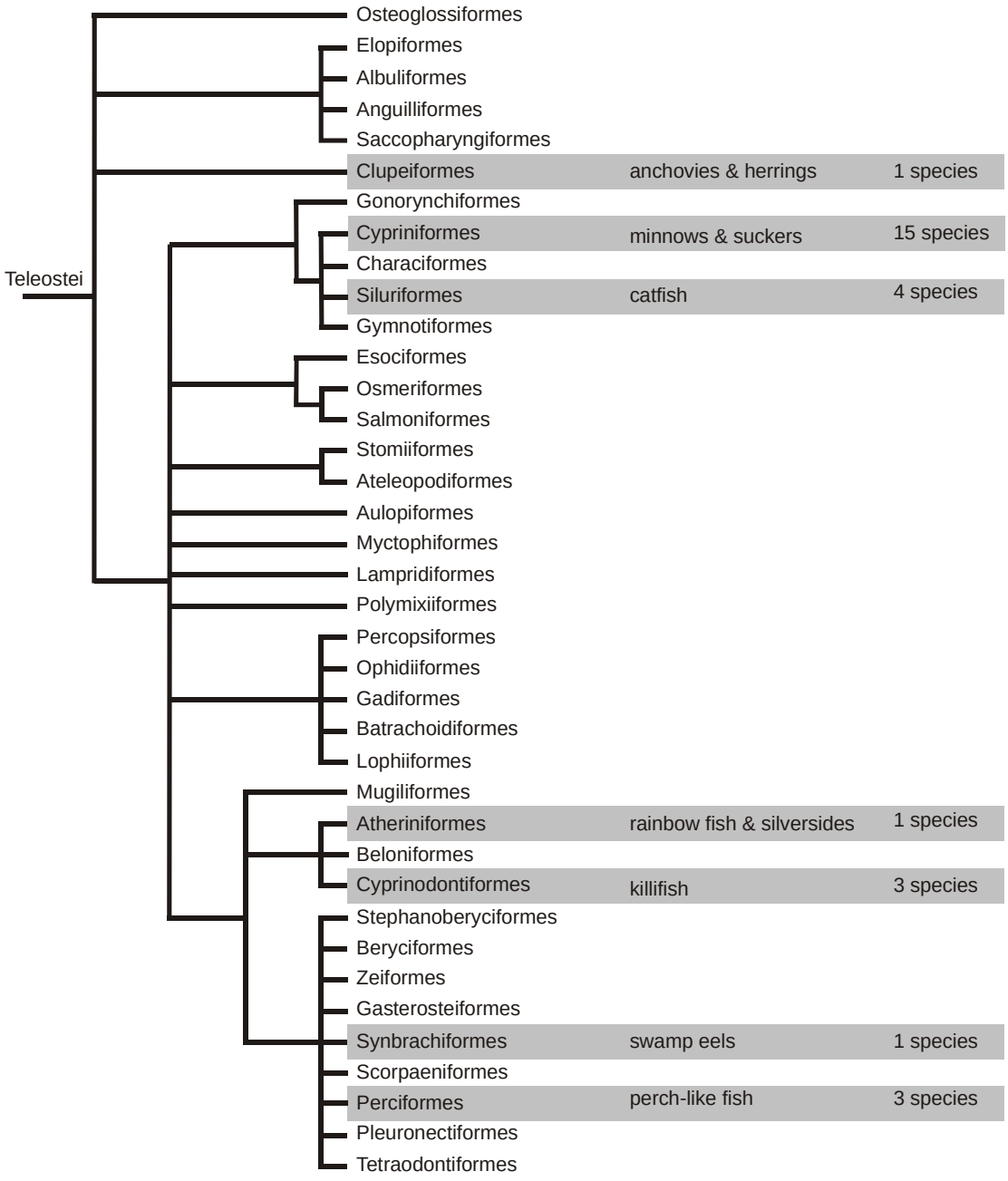

Figure 1. Phylogenetic tree of teleost orders showing the distribution of renal thyroid heterotopia within the Teleostei infraclass. Number of fish species reported to exhibit renal thyroid heterotopia per order are shown in grey boxes. Adapted from Nelson, J. S. 2006, Fishes of the World. 4th ed. John Wiley & Sons, New Jersey, USA.

HPT- and HPI-axis interrelationships

Peripheral interactions

The teleostean head kidney can be considered to be analogous to the mammalian adrenal gland, as it is the unique site of the production of hormones that are involved in the stress response. Norepinephrine and epinephrine are released from chromaffin cells upon stimulation by the sympathetic nervous system, whereas cortisol release from interrenal cells is primarily stimulated by neuroendocrine signals (ACTH). The work presented in this thesis focussed on putative interactions between the heterotopic thyroid follicles and the interrenal cells.

Static incubation of head kidney tissue with exogenous cortisol stimulated the release of thyroid hormones (**chapter 5**). Vice versa, neither short- nor long-term static incubation of head kidney tissue with thyroid hormones had any effect on the release of cortisol (**chapter 4**). Remarkably, ACTH stimulated the release of thyroid hormones from head kidney fragments (**chapter 5**). This ACTH effect could be mediated via endogenous cortisol. The thyroid stimulating effects of cortisol and ACTH indicate a paracrine interaction within the head kidney, although an endocrine interaction cannot be excluded. Further studies on glucocorticoid receptor expression should clarify this. As the cortisol concentrations applied are in the range of physiological plasma cortisol concentrations we exclude pharmacological effects. The responsive concentration of cortisol (1000 nM) (**chapter 5**) corresponds with plasma concentrations (500-1300 nM) measured in stressed carp (Huising, et al. 2004; Metz, et al. 2005). It may very well be that the observed interaction of cortisol with thyroid follicles is functional only during an activated stress response and not in stress-free conditions.

Perhaps the strongest argument for an endocrine rather than a paracrine mechanism for the effect of cortisol on thyroid hormone release in head kidney, is the stimulation of thyroid hormone release by exogenous cortisol and ACTH in kidney tissue (**chapter 5**), a tissue devoid of interrenal cells. Either endocrine or paracrine, the effects of cortisol and ACTH on the release of thyroid hormones in

the renal tissues, allow for an unprecedented unidirectional stimulatory interaction of the HPI-axis with the HPT-axis.

Central interaction

Apart from peripheral interactions between the HPI- and HPT-axis, interactions also occur centrally, *viz*. in the hypothalamic preoptic nucleus in common carp. Via *in vitro* and *in vivo* studies we established that thyroid hormones have a modulating effect on the hypothalamic CRH system. While most components of the CRH-system, *viz*. UI, CRH, CRH-R1 and CRH-R2, are relatively unaffected by thyroid hormones, CRH-BP mRNA expression is stimulated both *in vitro* (**chapter 5**) as well as *in vivo* (**chapter 4**). It is generally assumed that by binding CRH, CRH-BP will decrease the amount of bio-available CRH, resulting in a decreased release of ACTH and cortisol. Indeed, the increased expression of CRH-BP in hyperthyroid carp coincides with decreased levels of plasma cortisol, indicating a systemic down-regulation of the HPI-axis (**chapter 4**).

Thus, two anatomical sites for the communication between the HPT- and the HPI-axis are found in common carp, namely the renal tissues and the nucleus preopticus. Peripherally, a unidirectional, synergistic communication is observed in which elevated levels of plasma cortisol in plasma could stimulate the release of thyroid hormones. Centrally the interaction is also unidirectional but antagonistic, in which increased levels of plasma thyroid hormones affect the hypothalamic CRH-system which will eventually result in a down-regulation of plasma cortisol. Combined, the two unidirectional interactions at the peripheral and central level constitute a bidirectional interplay between the HPT- and HPI-axis. Centrally, the HPT-axis inhibits the HPI-axis, whereas peripherally the HPI-axis stimulates the HPT-axis, forming an inter-neuroendocrine axis negative feedback system. The functional consequences of these HPT-/HPI-axis interactions will be discussed in the following paragraph.

Physiological relevance of the HPI- and HPT-axis interrelationship

Since both cortisol and thyroid hormones are implicated in the regulation of energy and general metabolism, we propose that this is the physiological context in which both the HPI- and the HPT-axis interact (**chapter 4 and 5**). To further develop this concept, the involvement of cortisol and thyroid hormones in energy metabolism is here discussed. It does not suffice to only address the anabolic and catabolic effects of cortisol and thyroid hormones on carbohydrate, lipid and protein metabolism, also the implementation of these effects in the context of energy redistribution for immediate use, e.g. early stress response, and long-term processes such as somatic growth have to be taken into account.

Energy redistribution

During a stress response or nutritional challenge a fast redistribution of energy is required. A stress response requires the mobilisation of energy sources, of which carbohydrates are the primary substrate. During starvation, energy sources are also mobilised: first carbohydrates, then lipids and eventually protein stores.

The catabolic and anabolic effects of cortisol on these three energy carriers in fish are well studied (Mommsen, et al. 1999). Best known is its effect on carbohydrate metabolism. During a stress response cortisol not only stimulates the utilisation of glucose; it also stimulates gluconeogenesis (Janssens and Waterman 1988; Mommsen, et al. 1999). Indeed, in fish, as in all vertebrates, cortisol stimulates a multitude of enzymes that are involved in glucose synthesis (Mommsen, et al. 1999). The effects of cortisol on lipid metabolism in fish are catabolic, as evidenced by decreased levels of triacylglycerol and phospholipids and increased levels of lipase activity and plasma free fatty acids (FFA) following cortisol administration (Dave, et al. 1979; Lidman, et al. 1979; Minick and Chavin 1969; Sheridan 1986).

The experimental data on the effects of thyroid hormones on carbohydrate and lipid metabolism in fish are less clear and considerable inter-experimental and inter-species differences have been reported (Plisetskaya, et al. 1983). Generally,

actions similar to those of cortisol can be assumed. Thyroid hormones have catabolic as well as anabolic effects on carbohydrate metabolism in fish, *viz*. glycogenesis and glycogenolysis (Plisetskaya, et al. 1983). A lipolytic effect of thyroid hormones in fish can be inferred from the increased plasma FFA levels and decreased lipid content in various tissues following thyroid hormone administration (Barrington, et al. 1961; Murat and Serfaty 1970; Narayansingh and Eales 1975; Singh 1979; Takashima, et al. 1972).

Although these data suggest a role for thyroid hormones in the regulation of carbohydrate and lipid metabolism similar to that of cortisol, it remains to be investigated whether thyroid hormones play a permissive role in cortisol actions and the regulation of the stress response. The *in vitro* effect of cortisol on the release of thyroid hormones (**chapter 5**) is a first indication that the peripheral interaction of the HPI- with the HPT-axis is indeed involved in the regulation of a stress response.

However, during fasting the interaction between the HPI- and the HPT-axis appears not to be involved in the initial mobilisation and redistribution of energy. In fasted carp, plasma glucose and FFA already reach minimum and maximum levels, respectively, after 24 hours and these levels are sustained throughout a 6-weeks fasting period (Huising, et al. 2006). Plasma fT3 levels and renal ORD activity however, show a gradual decline during the 6 weeks fasting period, only reaching a maximum effect after 4 weeks (**chapter 7**). Although we did not measure thyroid hormone plasma levels and renal ORD activity in the first 2 weeks after fasting, it appears that thyroid hormones, and therefore the HPI- and HPT-axis interactions, are not involved in the immediate shift of a carbohydrate to a lipid metabolism in common carp upon fasting.

Somatic growth

Cortisol and thyroid hormones are not only involved in the process of energy redistribution, but they also affect somatic growth. Growth is the net difference between the incorporation of energy, *viz*. assimilation of lipids and proteins, and

energy expenditure, *viz*. break down of carbohydrates, lipids and proteins. Since growth and weight gain in fish is mainly established by protein (muscle) assimilation, we will focus on the effects of both hormones on protein metabolism and growth in general.

In fish, elevated levels of cortisol (as experienced during a stress response) exert catabolic effects on protein metabolism and reduce growth. A stress response not only increases plasma amino acid levels, indicating proteolysis, but in the long term also induces weight loss in a number of teleost species (Andersen, et al. 1991; Mommsen, et al. 1999; Pickford, et al. 1970; Storer 1967; Vijayan, et al. 1997). The effects of thyroid hormones on protein metabolism in fish are less clear, and catabolic as well as anabolic effects have been reported (for review see Plisetskaya, et al. 1983). In studies were exogenous thyroid hormones were administered the effects depended on the dose and mode of administration. Despite the equivocal results, a consensus exists that low doses of thyroid hormones stimulate protein anabolism and that this effect is physiologically relevant. Less controversial is the stimulatory effect of thyroid hormones on somatic growth in fish. As discussed in **chapter 7**, plasma levels of thyroid hormones, in particular the bioactive T3, and peripheral deiodinase activity are strongly and positively correlated with growth rate in various fish species, including common carp.

The opposite effects of cortisol and thyroid hormones on growth are further illustrated by their effects on the growth hormone (GH)/insulin like growth factor I (IGF-I) system, which plays a pivotal role in the regulation of growth (Moriyama, et al. 2000; Rousseau and Dufour 2007; Wood, et al. 2005). In several teleostean fishes, including common carp, thyroid hormones have been shown to stimulate the expression of GH and IGF-I, both *in vivo* and *in vitro* (Farchi-Pisanty, et al. 1995; Leung, et al. 2008; Luo and Mckeown 1991; Moav and Mckeown 1992; Schmid, et al. 2003). Administration of cortisol in several fish species inhibited the expression and release of IGF-I and stimulated that of IGF-I binding protein (Leung, et al. 2008; Peterson and Small 2005; Pierce, et al. 2006).

The antagonistic effects of cortisol and thyroid hormones on protein metabolism and somatic growth in fish may indeed form a physiological context for the existence of the HPI- and HPT-axis interactions in common carp. The

interplay between both systems is reflected in the process of long-term energy redistribution, *viz*. incorporation of energy and subsequent somatic growth, rather than the immediate redistribution of energy following fasting. The regulation of thyroid status, and thus growth, does not only involve the activity of the HPT- and HPI-axis, other neuroendocrine systems such as the melanocortin system are implicated as well, as will be discussed in the next paragraph.

HPT-axis and melanocortin-system interrelationship

Classically, the vertebrate thyroid gland is stimulated by the pituitary hormone TSH, which is stimulated by hypothalamic TRH. Both TSH and TRH cells are inhibited by thyroid hormones, to form a HPT-negative feedback loop. Also in common carp, thyroid hormone release is stimulated by TSH (**chapter 2**) and TSH expression is inhibited by thyroid hormones (**chapter 5**). Hypothalamic TRH, however, is not involved in the stimulation of thyroid gland activity via TSH in common carp; despite its expression being stimulated by thyroid hormones, TRH itself has no effect on the expression of TSH (**chapter 5**). In contrast to coho salmon (*Oncorhynchus kisutch*) and other vertebrate species where CRH rather than TRH may be the prime thyrotropin regulator (De Groef, et al. 2006; Larsen, et al. 1998), in common carp CRH did not affect TSH expression (**chapter 5**). We are therefore left to describe the hypothalamic factor that is involved in the regulation of carp pituitary TSH release. However, our work does not allow the definitive conclusion that TRH and CRH are not thyrotropic in common carp. The observation that both ACTH and α-MSH stimulates the release of thyroid hormones (**chapter 5**) *does* establish CRH and TRH as thyrotropic hormones, for these factors are the main hypothalamic melanotrope and corticotrope stimulating factors in common carp (Metz, et al. 2004; van den Burg, et al. 2005). The interrelationship of the melanocortin system and the thyroid system is also observed *in vivo*. Hyperthyroidism leads to increased levels of plasma α-MSH. Furthermore, the expression of pituitary POMC and PC1, which are key for the synthesis of α-MSH, was increased by thyroid hormone *in vivo* (**chapter 4**) as well *in vitro* (**chapter 5**).

Although circulating α-MSH has been implicated in several physiological processes, e.g. the immune response, reproduction and lipolysis, no single clear function of plasma α-MSH has been established in teleostean fishes (Flik, et al. 2006; Metz, et al. 2005). The pleiotropy of α-MSH can be inferred from the widespread distribution of its five main receptors (Metz, et al. 2006; Takahashi and Kawauchi 2006). Indirect evidence suggested a mild corticotropic role for α-MSH during chronic stress in Mozambique tilapia (Balm, et al. 1995; Lamers, et al. 1992). Although in common carp plasma levels of α-MSH are elevated during a stress response, as in other fish species, synthetic α-MSH did not stimulate the release of cortisol *in vitro* (Metz, et al. 2005). It appears that α-MSH is not a general corticotropic factor in teleost fish.

The thyrotropic action of α-MSH, as described in this thesis, is a novel function for circulating α-MSH in teleost fish and may very well be the principal action of α-MSH in fish, considering its role in the regulation of energy metabolism. Besides the peripheral role of α-MSH in lipid metabolism and the release of the metabolically important thyroid hormones from renal tissues, α-MSH is involved in the central nervous system in the regulation of metabolic processes as well. In mammals as well as in fish, central α-MSH provides anorexic signals to specific regions and nuclei, where it counteracts the orexigenic input of NPY/AgRP neurons to regulate food intake (Cerdá-Reverter, et al. 2003; Forlano and Cone 2007; Schwartz, et al. 2000). The central and peripheral involvement of α-MSH in metabolic processes, *viz*. the regulation of food intake and release of the thyroid hormone respectively, positions α-MSH as a key metabolic hormone in fish. It may not be surprising that some of the oldest and best conserved endocrine signals (NPY, CRH and POMC-derived peptides) play such important roles in food handling, a process that lies at the root of heterotrophic physiology.

Integrative neuroendocrine control of thyroid gland activity

Clearly, in common carp the neuroendocrine systems that are involved in the release of cortisol, thyroid hormones and α-MSH form no individual and parallel-running negative feedback system. The integration of these neuroendocrine signals, as described in this thesis are summarised in figure 2 and 3. A highly integrated hypothalamo-pituitary-interrenal/thyroid system can be constructed. This system is conceptually different and more complex than the classical HPI- and HPT-axis as described in the introduction. Although the representation of these regulatory systems as parallel-running negative feedback axes has clear conceptual value, it does not represent the *in vivo* situation.

When we subdivide the different endocrine signals that have been investigated in this thesis into stress (CRH-ACTH-cortisol), thyroid (TSH-thyroid hormones) and melanocortin (TRH-α-MSH) components (Fig. 3B), three other, albeit not mutually exclusive, neuroendocrine mechanisms for the regulation of thyroid gland activity in the common carp become apparent:

I. A short-loop negative feedback system, consisting of pituitary TSH and thyroid hormones.
II. A positive feedback system, consisting of hypothalamic TRH, pituitary α-MSH and thyroid hormones.
III. A negative feedback system, consisting of hypothalamic CRH, pituitary ACTH, interrenal cortisol and thyroid hormones.

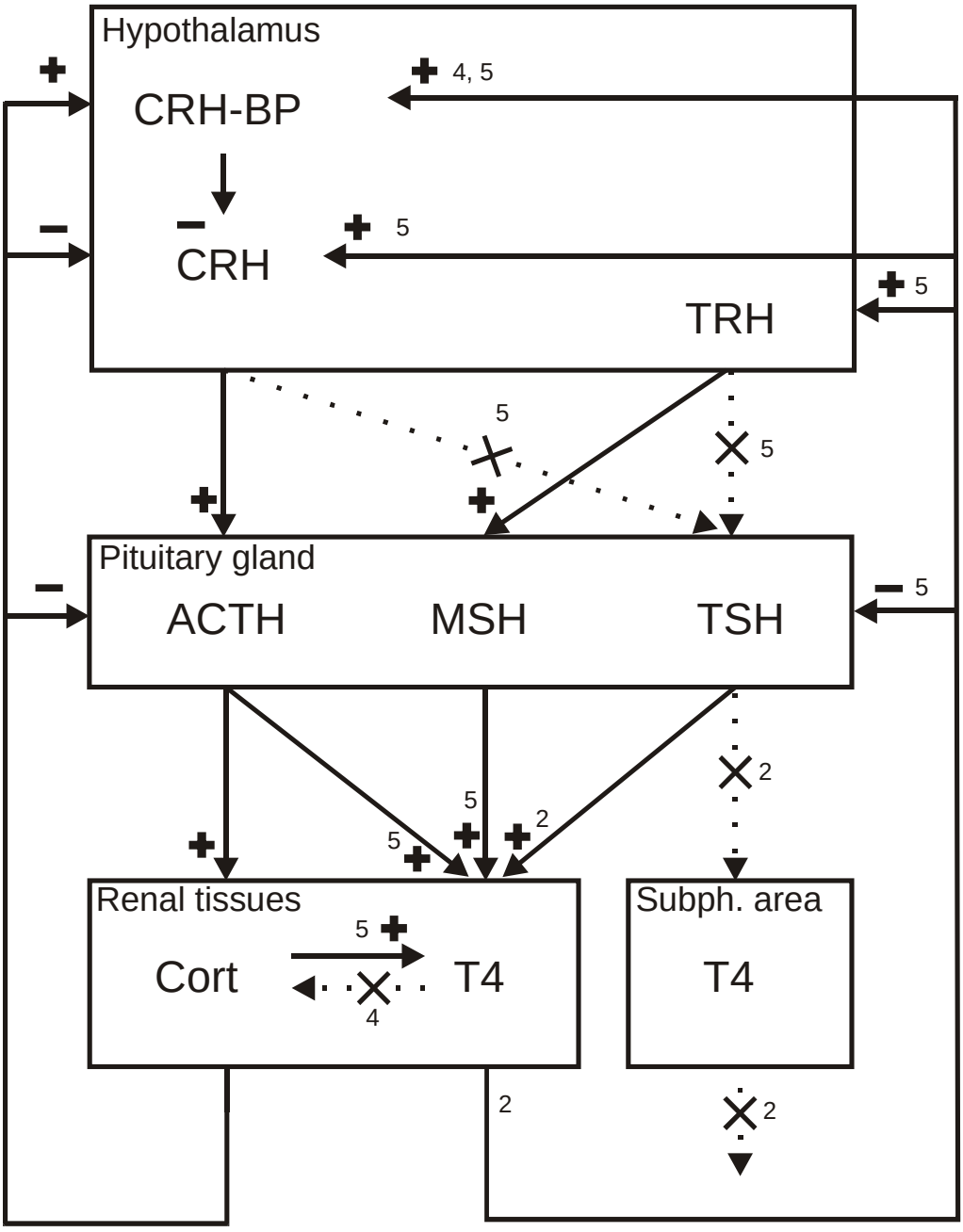

Figure 2. Schematic overview of the integration of the neuroendocrine thyroid-, stress- and melanocortin systems in common carp. The numbers refer to the chapters in this thesis where the specific effect or lack of effect is described. Arrows represent a stimulating effect, T-bars represent an inhibitory effect and dotted, crossed arrows represent a lack of effect.

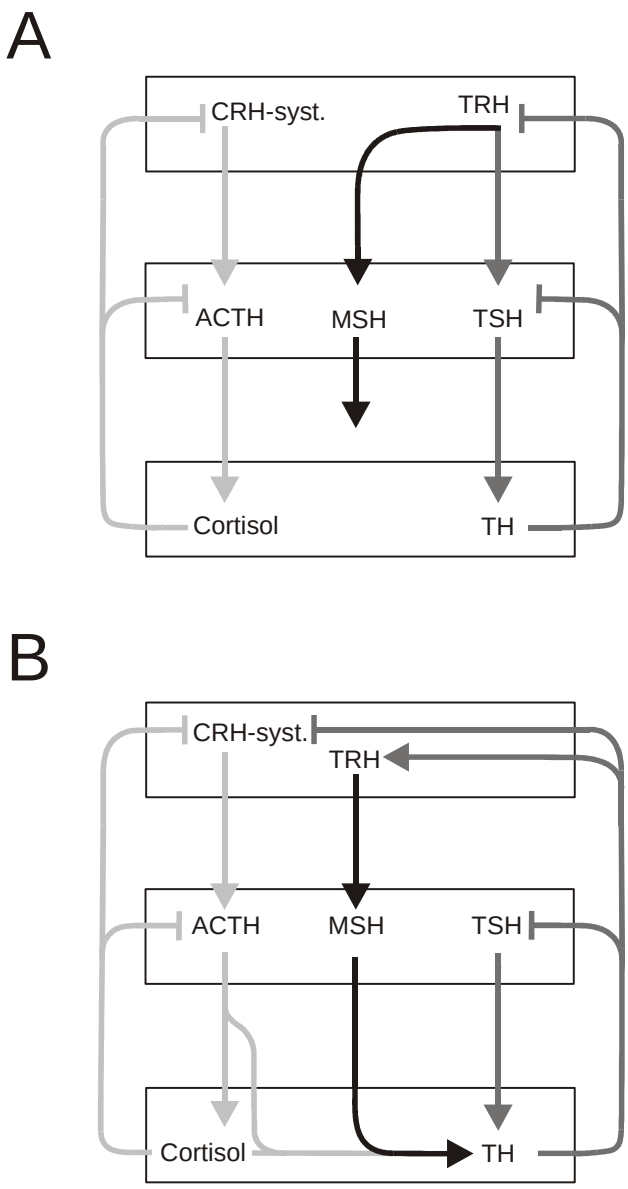

Figure 3. A. Representation of the classical thyroid-axis (grey), stress-axis (light grey) and melanocortin system (black) in common carp. **B.** Representation of the interrelationships of the three neuroendocrine systems as described in this thesis, revealing three alternative mechanisms for the regulation of thyroid gland activity and establishing a neuroendocrine web with participation of (shared) components of the thyroid-axis, stress-axis and melanocortin system. CRH-syst. = CRH-system, including CRH and CRH-BP; TH = thyroid hormones.

These three neuroendocrine pathways allow for a high degree of flexibility in the regulation of thyroid gland activity. It also allows for the integration of a multitude of inputs that are mediated via CRH, TRH, ACTH, α-MSH, TSH and cortisol on the regulation of thyroid functioning. The exact physiological relevance and relative importance of each mechanism on the total activity of the thyroid gland remains to be investigated.

Perspectives

The concept of parallel-running hypothalamic-pituitary-peripheral target axes is not only oversimplified, it also implies that these axes are confined between the level of the hypothalamus and the peripheral endocrine target tissues, *viz*. the interrenal cells and thyroid follicles. To understand the exact physiological relevance of the integrative aspects of the described neuroendocrine web, we have to extend the perspective of this web both up-stream as down-stream.

Downstream of the endocrine target tissues, processes like deiodination, conjugation and binding to plasma proteins determine the bioavailability and activity of thyroid hormones and, to some extent, that of cortisol. Furthermore, the specific expression of their respective receptors determines the targeting and functioning of these hormones. There are also several indications that at the level of these peripheral processes, components of the systems that regulate stress responses and thyroid status communicate. For instance, in Senegalese sole (*Solea senegalensis*) cortisol administration resulted in a tissue specific regulation of deiodinase activity, and in rainbow trout the expression of hepatic thyroid hormone receptor α-subunit is down regulated upon cortisol treatment (Aluru and Vijayan 2007; Arjona, et al. in prep.).

The hypothalamus is the principal integration centre for central signals, which are then conveyed to neuroendocrine signals as TRH and CRH. To understand which external stimuli induce the activation of the neuroendocrine web as described in the previous chapter, it is necessary to understand which signals from higher brain centres are integrated in the hypothalamic TRH- and

CRH-producing neurons. Retrograde neuron tracing techniques could be employed to identify the brain regions that are connected to the subsets of hypothalamic TRH- and CRH-producing neurons.

Extending the perspective of a neuroendocrine web beyond the level of the hypothalamus and endocrine tissues is crucial for understanding the exact physiological relevance of such a web. This approach will further complete the neuroendocrine web as it incorporates its input, external stimuli, and its output, functioning and targeting of cortisol and thyroid hormones. However, the mere description of additional interactions within this neuroendocrine web does not suffice, it requires a functional analysis of its dynamics to fully appreciate these interactions within a physiological context.

We therefore propose to perform, apart from descriptive analyses, additional functional studies of the interactions of the neuroendocrine web within physiological paradigms beyond that of hyperthyroidism (**chapter 4**), e.g. hypothyroidism, stress response or nutritional challenge.

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Samenvatting in het Nederlands (Summary in Dutch)

De onderzoeken beschreven in dit proefschrift omvatten verschillende aspecten van het schildkliersysteem van de karper (*Cyprinus carpio*). Niet alleen is de schildklier zelf onderzocht, ook de aansturing van de schildklier vanuit de hersenen, de interactie met het stress-systeem en de enzymatische omzettingen van het schildklierhormoon zijn belicht. De vergaarde resultaten zijn vergeleken met die in andere vissoorten en vertebraten. Uit een dergelijke vergelijkingen kunnen nieuwe mechanismen en concepten binnen de schildklierfysiologie worden herleid. Ook vanuit het perspectief van aquacultuur is schildklieronderzoek aan de karper interessant, aangezien de kweek van karperachtigen 45% van de totale, wereldwijde aquacultuur omvat en schildklierhormonen fundamentele biologische functies vervullen. Dit onderzoek kan derhalve een bijdrage leveren aan een verbeterde aquacultuur. Schildklierhormoon is betrokken bij een grote verscheidenheid aan fysiologische processen. In zijn algemeenheid omvatten de functies van schildklierhormoon de regulatie van ontwikkelingsprocessen (o.a. metamorfose), groei en metabole processen (o.a. het basaal metabolisme en warmteproductie).

Locatie en activiteit van de schildklier

De synthese van schildklierhormoon vindt plaats in schildklierfollikels. Deze bolvormige structuren bestaan uit een extracellulaire eiwitmatrix, het colloid, omsloten door een enkele laag van schildkliercellen. Uniek aan het schildklierhormoon is de incorporatie van jodide. Jodide wordt actief opgenomen door de schildkliercel en getransporteerd naar het colloid, waar het wordt gekoppeld aan tyrosine-residuen in het eiwit thyroglobuline. Na verschillende enzymatische processen, gelokaliseerd op het grensvlak van de schildkliercel en het extracellulaire colloid, worden hieruit schildklierhormonen gesynthetiseerd. De driedimensionale follikelstructuur lijkt dus essentieel voor de vorming van schildklierhormoon. De twee belangrijkste schildklierhormonen zijn thyroxine, T4 (dat vier jodide atomen bevat) en triiodothyronine, T3 (drie jodide atomen). In zoogdieren vormen de

schildklierfollikels een compacte schildklier die gelegen is in de hals. In vissen vormen de schildklierfollikels echter geen compacte schildklier, maar liggen de schildklierfollikels los verspreid in het onderkaak- of subpharyngeale-gebied. In sommige vissoorten, met name karperachtigen, zijn schildklierfollikels ook waargenomen in andere anatomische locaties, met name in nierweefsel zoals de kopnier en de nier. In **hoofdstuk 2** zijn de locatie en activiteit van de schildklier in karper en tilapia (*Oreochromis mossambicus*) onderzocht. De locatie van de schildklier in tilapia is typisch voor vissen aangezien schildklierweefsel alleen in het onderkaakgebied aanwezig is. In de karper bleek de actieve schildklier zich echter geheel in het nierweefsel te bevinden. De actieve opname van jodide, alsmede de synthese en secretie van schildklierhormoon vonden alleen hier, in de kopnier en nier, plaats en niet in het onderkaakgebied. Met name de nier, die bijna 90% van de totale hoeveelheid schildklierfollikels bevat, kan worden beschouwd als de locatie van de ware endocriene schildklier in de karper. Ondanks dat in het onderkaakgebied van de karper geen actieve jodide-opname, schildklierhormoonsynthese of -secretie plaatsvindt, bevindt zich er toch een aanzienlijke hoeveelheid schildklierfollikels (10% van het lichaamstotaal) waarin T4-immunoreactiviteit aanwezig is. Klaarblijkelijk zijn deze, nu inactieve follikels, ooit, tijdens een eerdere levensfase, actief geweest en in staat schildklierhormoon te synthetiseren, en is deze functie later overgenomen door schildklierfollikels in nierweefsel. Uit onderzoek beschreven in **hoofdstuk 3** blijkt inderdaad dat tijdens de eerste maand na het uitkomen uit het ei, er geen schildklierfollikels of T4-immunoreactiviteit in de kopnier of nier aanwezig is, terwijl er in de onderkaak al na vier dagen T4-immunoreactiviteit detecteerbaar is. Deze is aanvankelijk alleen in losse cellen en celgroepen te zien, pas na een maand ontwikkelen zich de eerste schildfollikels. Blijkbaar is het schildklierweefsel in de onderkaak verantwoordelijk voor de vroege schildklierhormoonproductie en is deze onafhankelijk van een driedimensionale folliculaire structuur. Onduidelijk is nog wat de ontwikkeling van schildklierweefsel in nierweefsel initieert. Wellicht is het intieme contact en interactie met andere endocriene celtypen (bijvoorbeeld cortisolproducerende cellen in de kopnier) een reden voor deze translocatie.

Centrale regulatie van schildklier en interactie met stress systeem

Net als in andere vertebraten wordt in vissen de activiteit van de schildklier voornamelijk aangestuurd vanuit de hersenen via de zogenaamde HPT-as (*hypothalamo-pituitary-thyroid*-as). Vanuit de nucleus preopticus (NPO), een hersengebied in de hypothalamus, projecteren *thyrotropin-releasing hormone* (TRH) neuronen op thyrotrope cellen in de hypofyse (*pituitary gland*), een belangrijke endocriene klier gelegen onderaan de hersenen. TRH stimuleert de thyrotrope cel tot de secretie van het *thyroid-stimulating hormone* (TSH) dat de schildklier stimuleert tot de secretie van schildklierhormonen. De schildklierhormonen zelf hebben weer een negatief effect op de productie van TSH en TRH, en zo vormt zich een negatief terugkoppelingssysteem voor de regulatie van schildklieractiviteit. Een soortgelijk negatief terugkoppelingssysteem (de *hypothalamo-pituitary-interrenal*-as; HPI-as) bestaat voor het stresshormoon cortisol, dat wordt gesynthetiseerd in de interrenale cellen van de kopnier. In de HPI-as zijn de hypothalame en hypofysaire stimulerende factoren respectievelijk *corticotropin-releasing hormone* (CRH) en *adrenocorticotropic hormone* (ACTH). In verschillende vissoorten, waaronder de karper, heeft TRH echter geen effect op thyrotrope cellen, en is CRH mogelijk een alternatieve thyrotrope factor. In verschillende diersoorten, waaronder vogels, reptielen, amfibieën en vissen, is aangetoond dat CRH een stimulerend effect heeft op thyrotrope cellen. Een dergelijk tweeledig effect positioneert CRH als een signaalmolecuul dat betrokken is bij de integratie en gecoördineerde werking van de HPT- en HPI-as. Verder wijst de aanwezigheid van schildklierfollikels nabij de cortisolproducerende interrenale cellen in de kopnier op een mogelijke integratie van beide systemen op een perifeer niveau.

In **hoofdstuk 4** is onderzocht of componenten van het stress-systeem in karper gevoelig zijn voor een verhoogde schildklierstatus (hyperthyreoïdie). De belangrijkste aanwijzing dat beide systemen interacteren is een verlaagde cortisol concentratie in het plasma van karpers die met T4 zijn geïnjecteerd. Deze verlaging gaat gepaard met een verhoogde expressie in de hypothalamus van een eiwit dat CRH bindt (CRH-BP) en daarmee de biologische activiteit van CRH moduleert. Hoewel de expressie van CRH onveranderd blijft, kan een verhoging van CRH-BP resulteren in een verlaagde concentratie van beschikbaar bio-actief CRH. Opvallend in deze hyperthyreote karpers is de

stimulatie van alpha-*melanocyte-stimulating hormone* (α-MSH) dat wordt geproduceerd in melanotrope cellen die onder positieve controle staan van TRH uit de hypothalamus.

Om een duidelijker beeld te krijgen van de verschillende interacties tussen de HPT- en HPI-as in de karper, zijn in **hoofdstuk 5** een aantal *in vitro* experimenten beschreven. Uit de resultaten hiervan blijkt dat zowel TRH als CRH niet in staat zijn om de expressie van TSH te stimuleren. Ook bleek de positieve invloed van T4 op de expressie van CRH-BP, zoals ook waargenomen in een intact dier (beschreven in **hoofdstuk 4**), en tevens die van CRH en TRH. In de karperschildklier (kopnier en nier) blijkt dat cortisol een stimulerend effect heeft op de afgifte van T4. Tevens blijkt dat ACTH en α-MSH de afgifte van T4 stimuleren, waarmee twee nieuwe hypofysaire thyrotrope factoren, naast TSH (beschreven in **hoofdstuk 2**), in de karper worden beschreven.

Samengevat worden twee anatomische locaties beschreven waar een interactie plaatsvindt tussen beide systemen; de schildklier in de periferie en de NPO in de hersenen. De schildklier zelf wordt gestimuleerd door het stress-systeem, terwijl in de NPO het schildkliersysteem het stress-systeem inhibeert via de stimulatie van CRH-BP. Een mogelijke fysiologische context voor deze interacties is de regulatie van metabole processen, aangezien zowel schildklierhormoon als cortisol hierbij betrokken zijn. Bij snelle metabole processen als de redistributie van energie, bijvoorbeeld tijdens vasten of een stressrespons, lijken de effecten van beide hormonen synergistisch, terwijl bij langzame metabole processen als groei de effecten van beide hormonen juist antagonistisch lijken.

Deze onderzoeken laten zien dat de schildklier in de karper niet wordt gereguleerd door een typische "klassieke" HPT-as. In de karper zijn drie factoren uit de hypofyse beschreven die de secretie van schildklierhormoon uit de schildklier stimuleren, namelijk TSH, ACTH en α-MSH. Ondanks dat CRH en TRH geen effect hebben op TSH, zijn deze hormonen indirect toch betrokken bij de regulatie van de schildklieractiviteit, aangezien zij hoofdverantwoordelijk zijn voor de stimulatie van ACTH en α-MSH secretie. Uit deze gegevens kunnen drie routes voor de regulatie van de schildklieractiviteit worden herleid. Eén route gemedieerd via TSH, een tweede via TRH en α-MSH en een derde via CRH en ACTH. Door de veelvoud aan neuroendocriene signalen en

variaties in deze drie routes, is een hoge mate van flexibiliteit en integratie mogelijk bij de regulatie van de schildklieractiviteit. Hieruit blijkt dat de beschrijving van de regulatie van schildklieractiviteit door middel van één enkele HPT-as slechts conceptionele waarde heeft en duidelijk geen reflectie is van de *in vivo* situatie.

Dejodase activiteit

De activiteit van het schildkliersysteem wordt niet alleen centraal gereguleerd, maar ook perifeer via metabole omzettingen van het schildklierhormoon, wat leidt tot verschillende schildklierhormoon-metabolieten met elk hun specifieke activiteit. Eén van deze processen is dejodering waarbij door een enzym uit de familie van de dejodases een jodide-atoom van het schildklierhormoon wordt verwijderd. Aangezien de schildklier hoofdzakelijk het inactieve prohormoon T4 produceert, is de perifere omzetting van T4, via dejodering, naar het bio-actieve T3 bepalend voor de perifere schildklierstatus. Dejodase-activiteiten bepalen niet alleen de T3-concentratie in het (bloed)plasma, maar ook die in het intracellulaire cytoplasma. Zo kunnen dejodases, onafhankelijk van de schildklieractiviteit, op lokaal niveau, per weefsel de beschikbaarheid van T3 bepalen. In vertebraten zijn drie dejodase-isovormen gekarakteriseerd, aangeduid met D1, D2 en D3, en elk met een eigen schildklierhormoonaffiniteit, weefseldistributie en activerende of inactiverende activiteit. Dejodases in vissen komen qua structuur en biochemie grotendeels overeen met die in zoogdieren, al zijn er echter enkele opmerkelijke biochemische verschillen. Zo wordt in sommige vissen de dejodase activiteit geïnhibeerd door de reducerende zwavelverbinding dithiothreitol (DTT), terwijl in zoogdieren DTT juist een activerende rol heeft.

In **hoofdstuk 6** is een biochemische en enzymkinetische analyse beschreven van dejodases in de lever en nier van de karper. Terwijl de dejodase-activiteit in de nier wordt geremd door DTT, wordt die in de lever juist gestimuleerd. Verder bestaat de dejodase-activiteit in de nier uit twee enzymatisch actieve componenten, en die in de lever uit slechts één, met bovendien een bijzondere substraat-inhibitie kinetiek. Hiermee zijn voor het eerst binnen één diersoort dergelijke weefselspecifieke verschillen in de

biochemie en kinetiek van dejodases beschreven. Deze verschillen kunnen echter vooralsnog niet worden gerelateerd aan de aanwezigheid van een specifieke dejodase isoform.

Zoals eerder aangegeven zijn schildklierhormonen betrokken bij verschillende metabole processen. Zo wordt de activiteit van het schildkliersysteem bepaald door de voedselinname. In **hoofdstuk 7** is de perifere regulatie van het schildkliersysteem door dejodases onderzocht in relatie tot voedselinname. In karpers die gedurende zes weken vastten, daalde de plasma T3 concentratie terwijl die van T4 onveranderd bleef. Deze daling correleert met een gedaalde dejodase-activiteit en expressie van D2 in de nier, wat erop wijst dat dit enzym in dit orgaan verantwoordelijk is voor de regulatie van circulerend T3 tijdens vasten. In vissen die onbeperkt voedsel hebben gekregen, zien we dat het perifere schildkliersysteem nauwelijks is beïnvloed, ondanks een twee tot vier maal hogere voedselinname. Opvallend is dat ondanks deze hogere voedselinname de groeisnelheid van de vissen ook niet is veranderd. Hieruit blijkt dat de activiteit van het perifere schildkliersysteem in de karper correleert met groei, en niet zozeer met voedselinname. De langzame respons van het schildkliersysteem tijdens het vasten (een maximaal effect werd bereikt na vier weken vasten) is een andere aanwijzing dat tijdens vasten het schildklierhormoon betrokken is bij langzame metabole processen, zoals groei, en niet bij snelle metabole processen, welke al binnen 24 uur na vasten plaatsvinden.

De onderzoeken in dit proefschrift beschrijven enkele mechanismen en concepten in de schildklierfysiologie van de karper die nog niet eerder zijn beschreven in andere vertebraten. Zo bevindt de complete endocriene schildklier zich in nierweefsel, is de schildklierhormoon-synthese aanvankelijk intracellulair, beïnvloedt schildklierhormoon het hypothalame CRH-systeem via CRH-BP, stimuleren cortisol, ACTH en α-MSH de afgifte van schildklierhormoon en verschillen dejodases uit twee weefsels binnen één diersoort in biochemie, enzym-kinetiek en hun activiteit tijdens vasten. Hieruit blijkt dat de karper in het bijzonder, en vissen in het algemeen, waardevolle modelsystemen kunnen zijn voor onderzoek binnen de vergelijkende schildklierfysiologie.

Dankwoord (Acknowledgements)

"En, heb je dat boekje al af?" Hoe vaak ik dit de afgelopen jaren wel niet heb moeten aanhoren en hoe vaak heb ik niet geantwoord met "Ja, bijna…", met een zekere twijfel in mijn stem. Maar nu, nu kan ik eindelijk zeggen dat het klaar is. Dat heb ik natuurlijk niet alleen aan mezelf te danken, hiervoor ben ik heel veel mensen, heel erg dankbaar. Niet alleen op wetenschappelijk gebied ook zeker daarbuiten. Mijn periode op de afdeling Organismale Dierfysiologie was echt een unieke ervaring, vanwege de vele leuke en leerzame momenten maar ook door de bijzondere mensen die ik heb ontmoet.

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Curriculum vitae

Edwin Jacobus Wilhelmus Geven werd op 9 december 1977 geboren te Dinxperlo. In 1996 behaalde hij zijn VWO diploma aan het Isala College in Silvolde, waarna hij datzelfde jaar vertrok naar Nijmegen om de opleiding Biologie te volgen aan de toenmalig geheten Katholieke Universiteit Nijmegen. In de doctoraalfase van deze opleiding liep hij stage bij de afdeling Psycho-Neuro-Farmacologie (Prof. dr. A. R. Cools), bij de afdeling Organismale Dierfysiologie (Prof. dr. S. E. Wendelaar Bonga) en aan het Institute for Pharmacological Sciences van de Universitá degli Studi di Milano te Italië (Dr. B. A. Ellenbroek en Dr. M. Riva).

In 2002 wist hij de studie Biologie succesvol af te ronden, waarna hij in december dat jaar begon aan promotie onderzoek bij de afdeling Organismale Dierfysiologie onder begeleiding van Prof. dr. G. Flik en Dr. P. H. M. Klaren naar verschillende aspecten van de schildklierfysiologie en de mogelijke interacties met andere endocriene systemen in de karper. Tijdens deze periode is tevens een bijdrage geleverd aan de opleiding Biologie met de coördinatie van practica voor de cursus Dierkunde en het begeleiden van stages van Bachelor en Master studenten. De resultaten die tijdens het promotietraject werden behaald, zijn gepresenteerd tijdens verschillende internationale congressen en symposia en tevens gepubliceerd in verscheidene internationale wetenschappelijke tijdschriften.

Colour figures

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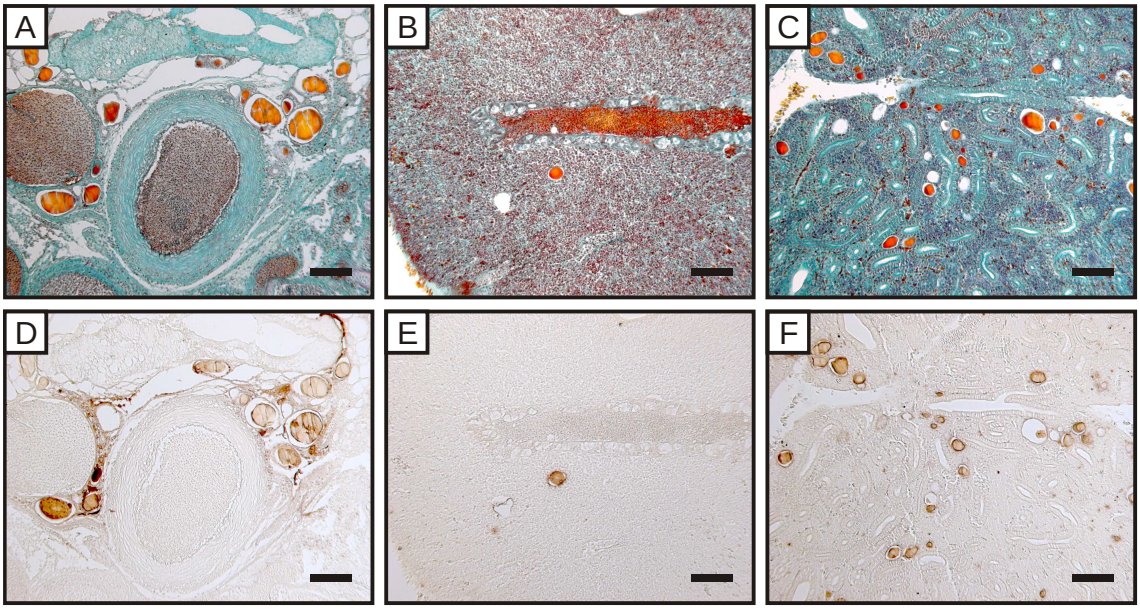

Figure 3. Crossmon's staining of 7 µm thick sections of carp subpharyngeal area (A), head kidney (B) and kidney (C). Thyroxine immunohistochemistry on serial sections of carp subpharyngeal area (D), head kidney (E) and kidney (F). Scale bars indicate 200 µm.

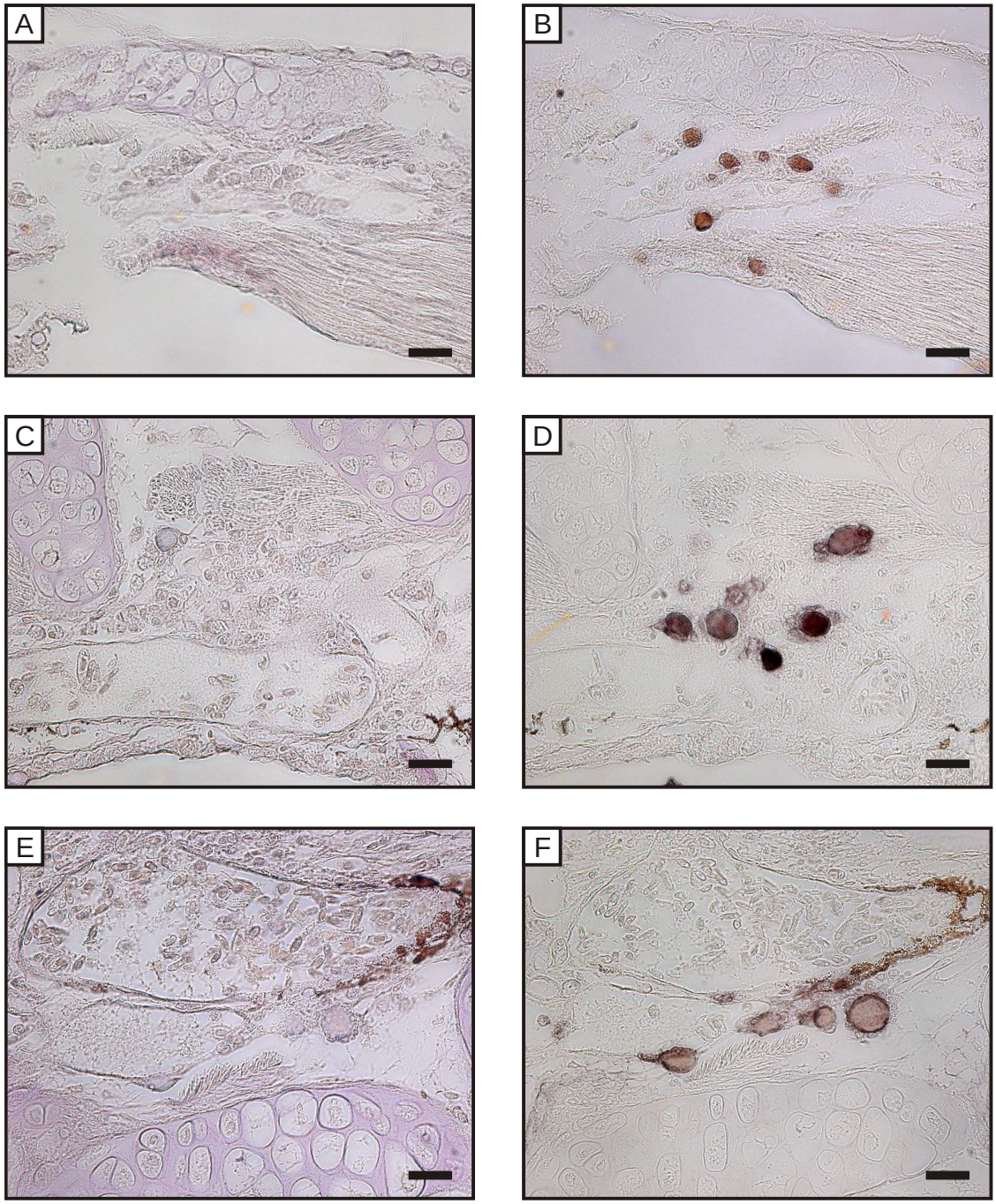

Figure 1. Crossmon's staining of 7 μm sections of the subpharyngeal area of carp at 8 (A), 20 (C) and 32 dph (E). Thyroxine immunoreactivity in adjacent sections of the subpharyngeal area of carp at 8 (B), 20 (D) and 32 dph (F). Scale bars indicate 20 μm.